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IMPACT OF THE SHORT-TERM CONSUMPTION OF A MODERATELY HIGH FAT DIET ON NITRIC OXIDE PRODUCTION AND BIOAVAILIBILITY

By Kan Huang

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ABSTRACT

IMPACT OF THE SHORT-TERM CONSUMPTION OF A MODERATELY HIGH FAT DIET ON NITRIC OXIDE PRODUCTION AND BIOAVAILIBILITY

By Kan Huang

Nitric oxide (NO) plays an essential role in the regulation of numerous biological processes. Its bioavailability is assured by a well regulated balance between NO generation and NO removal. Disruptions in this balance contribute to the pathogenesis of various diseases, including hypertension, Alzheimer's disease, diabetes mellitus and arthritis. Many factors contribute to the maintenance of NO bioavailability by controlling nitric oxide synthase (NOS) expression, NOS activity, the availability of substrates and cofactors involved in the generation of NO by NOS, levels of reactive oxygen species (ROS), and the formation and mobilization of NO reservoirs. Dietary factors have a significant impact on NO bioavailability. Of the major dietary constituents, fats have been the most extensively studied. The long-term consumption of high fat diets decreases NO bioavailability and induces some irreversible pathological changes in various organs of experimental animal models. The effect of the short-term consumption of excessive dietary fat is still unclear. The primary objective of the studies presented in this dissertation was to investigate the impact of the short-term consumption of a moderately high fat diet (MHFD) on NO generation and the mechanisms involved in the maintenance of NO bioavailability. The consumption of the MHFD markedly reduced urinary excretion of stable NO metabolites and levels of these metabolites in the plasma within a week of the onset of dietary treatment. eNOS expression was suppressed in a tissue-specific and

time-dependent manner. The earliest decrease in expression occurred in the liver at week one. In addition, hepatic NOS activity was depressed and nitrotyrosine levels were elevated; increased nitrotyrosine formation is indicative of the increased production of ROS. Other tissues in which NOS expression was suppressed in rats on the MHFD included the heart and kidney medulla.

In addition to affecting NO bioavailability, the ingestion of a MHFD also caused a decrease in drinking behavior. A portion of this reduction in drinking behavior may be attributable to the physical properties of the diet whereas the remainder is probably due to variations in the nutrient composition of the diet. The rats adapted to reduced drinking behavior by decreasing urine output and increasing urine osmolality. Nitric oxide synthase expression in the supraoptic and paraventricular nuclei did not change.

In conclusion, the short-term consumption of a MHFD has profound effects on circulating NO levels by affecting mechanisms that regulate NO availability in specific tissues. In addition, the ingestion of a MHFD may affect other biological functions such as drinking behavior.

DEDICATION

I would like to dedicate this work to all my family and friends who have supported me during my education.

To my grandmother, who is blessing me from heaven. Thank you for giving me the motivation of always looking forward no matter how hard the life is.

To my parents who lost the opportunities to receive their educations but supported my education and my dreams with everything they can do.

To my husband, Alex Wu for all your help and support in these years. I definitely cannot reach where I am standing today without you.

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Dr. Lawrence Grover, thank you for all your valuable suggestions to my research and all the funny things I have heard from you. I almost can say that I took another class from you which is “Live in America”.

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LIST OF SYMBOLS/NOMENCLATURE

AL - argininosuccinate lyase

AS - argininosuccinate synthetase

BH4 - tetrahydrobiopterin

CaM - calmodulin

cGMP - cyclic guanosine monophosphate

CpG - cytosine and guanine separated by a phosphate in DNA

Cys - cysteine

DNA - deoxyribonucleic acid

EDRF - endothelium derived relax factor

eNOS - endothelial nitric oxide synthase

FAD - flavin adenine dinucleotide

FMN - flavin mononucleotide

GSNO - S-nitrosoglutathione

GTPase - enzymes bind and hydrolyze guanosine triphosphate

Hb - hemoglobin

Hsp - heat shock protein

iNOS - inducible nitric oxide synthase

KDa - kilodalton

LDL - low-density lipoprotein

LPS - lipopolysaccharide

MAPK - mitogen-activated protein kinases

MHFD - moderately high fat diet

Mn - manganese

mRNA - messenger ribonucleic acid

NADPH - nicotinamide adenine dinucleotide phosphate

NAP110 - nitric oxide associated protein 110

NF-kappaB - nuclear factor-kappaB

nNOS- neuronal nitric oxide synthase

nM – nanomole

NMDA - N-methyl-D-aspartic acid

NO - nitric oxide

NOS - nitric oxide synthase

NOx - nitrite and nitrate

PDEs - cyclic nucleotide phosphodiesterases

PDZ - PSD-95/Dlg/ZO-1

PKG - cyclic guanosine monophosphate dependent protein kinase

ROS - reactive oxygen species

Ser - serine

sGC - soluble guanylyl cyclase

SNOs - *S*-nitrosothiols

TGF-*b* - transforming growth factor-*b*

TNF-α - tumor necrosis factor α

Tyr - threonine

US - United States

Vmax - maximum initial velocity of the enzyme catalysed reaction

PREFACE

There are four chapters in this dissertation, two of which contain primary research. Chapter I is a comprehensive literature review of the physiological role of nitric oxide and its regulation. Chapter II will be submitted for publication. It addresses the impact of the short-term consumption of a moderately high fat diet on nitric oxide production and bioavailability. Chapter III focuses on experiments that we have done to assess the impact of the ingestion of a moderately high fat diet on drinking behavior. The final chapter includes a general discussion of my research with conclusion and future directions.

CHAPTER I

Literature Review

(I) Nitric Oxide Introduction

In 1977, Murad discovered that nitroglycerin releases a compound which relaxes vascular smooth muscle (Katsuki et al. 1977). Later he identified this compound as nitric oxide (NO) (Arnold et al. 1978). In 1980, Furchgott demonstrated that a signaling molecule derived from the intact endothelium plays a critical role in promoting vasodilatation. Without knowing the chemical nature of this molecule, he named it endothelium derived relaxing factor (EDRF) (Furchgott and Zawadzki 1980). Eventually, Ignarro identified EDRF as NO in 1986 (Ignarro et al. 1986). The historic finding that NO is an endogenous signaling molecule was an important milestone in biomedical research. Because of their contributions in the discovery of NO as a key endogenous vasodilator, these three scientists won the Nobel Prize in Medicine in 1998. This was the first time that a gas was shown to function as a signaling molecule in an organism (Moncada et al. 1988; Moncada and Higgs 2006). Subsequently, NO-related research grew rapidly throughout the world. Due to the tremendous public attention NO attracted, it was chosen as the cover story by Science for its "Molecule of the Year" in 1992. By the end of 2008, there were more than 93,000 primary papers and 122,990 review papers published on NO research.

Biological characteristics of nitric oxide

NO is a highly reactive gas which is produced by a variety of tissues. Because of its extremely short half-life, it is thought to reach a target tissue by simple diffusion and act in a paracrine or autocrine fashion (Shin et al. 1992; Thomas et al. 2001); however, it has recently been shown that NO also can be transported as a molecular complex with specific blood proteins and released wherever it is needed (Kubes 2005; Gladwin et al. 2006). Due to its neutral and hydrophobic physical properties, NO can easily traverse cell membranes by simple diffusion (Denicola et al. 1996). It can be rapidly oxidized, reduced, or complexed to various biomolecules. The biological effects of NO are dependent upon multiple factors such as the local microenvironment in which NO is generated and the concentration of NO in the tissue (Yun et al. 1997).

The biological effects of NO can be separated roughly into direct and indirect effects (Ignarro et al.1986). If NO interacts directly with a specific biological molecule, the resulting biological effects are considered to be direct. The reaction of NO with certain metals is one of the best characterized direct effects. If an initial reaction generates a NO-derived intermediate that undergoes further reactions, then any effect mediated by the additional NO-derivatives are indirect effects. A classic example occurs when NO reacts with superoxide to produce peroxynitrite. The peroxynitrite, in turn, oxidizes the phenolic ring of tyrosine to form nitrotyrosine (Ignarro et al. 1986): any biological effects initiated by nitrotyrosine are considered to be indirect action of NO. In general, low concentrations of NO usually result in direct effects in normal tissues. High NO

concentrations are more likely to mediate indirect effects under pathological conditions (Grisham et al. 1999).

From a biological perspective, NO behaves in a paradoxical manner: on the one hand it functions as an essential signaling molecule; on the other hand, it is a highly reactive free radical. NO concentration appears to be a central factor in determining the ultimate effect of NO on a particular tissue. Many normal physiological functions require low concentrations of NO, but NO can react with other compounds when present at a high concentration to generate products that are toxic to cells (Grisham et al. 1999). For example, low physiological levels of NO promote nonspecific immunity that protects against viral or bacterial infection whereas elevated NO levels will produce an inflammatory response and cause tissue damage (Coleman 2001). Recent cancer research has shown apparently inconsistent findings regarding the effects of different levels of NO on tumor development and/or progression (Mocellin et al. 2007). Similar findings of paradoxical actions of NO were discovered in the cardiovascular system (Wever et al. 1998), central nervous system (Hlatky et al. 2003), and digestive system (Beck et al. 2004). These findings support the conclusion that a comprehensive and dynamic view should be taken when addressing the biological actions of NO.

The significance and complexity of the actions of NO are well established. NO has been shown to affect every major organ system. Moreover, more than 36 different functions have been described for this molecule (Gow 2006). The earliest discovered function of NO was in the cardiovascular system where it was shown to induce vasodilatation by stimulating smooth muscle relaxation through a cGMP-dependent mechanism (Ignarro et

al. 1986). The importance of NO in the regulation of vascular function has inspired many researchers to study diseases involving vascular tone alternation. It is widely accepted that NO deficiency plays a critical role in the development of essential hypertension (Vanhoutte and Boulanger 1995). By contrast, excessive NO production induces massive vasodilatation which causes severe hypotension in septic shock (Mitsuhata et al. 1995 ; Fernandes and Assreuy 2008). Another interesting paradoxical influence of NO on the cardiovascular system is that the low concentrations of NO generated by vascular endothelial cells prevent atherosclerosis; however, when NO is raised to a toxic level, it can promote the development of atherosclerosis (Dusting et al. 1998).

In the heart, NO plays an important role in regulating coronary blood flow. In addition to regulating coronary vascular tone, NO also affects cardiac structure and function. It affects not only large coronary arteries but also the cardiac microvasculature (Quyyumi et al. 1995). Experimental evidence suggests that NO may mediate an increase in diastolic fiber length and myocardiocyte distention, thereby contributing to left ventricular diastolic distension and reserve ventricular function (Petroff et al. 2001). Impaired NO function in cardiac remodeling after injuring the cardiac myocytes may lead to left ventricular dysfunction (Massion et al. 2003). If this condition persists, heart failure may result (Mohri et al.1997; Prasad et al. 2003). Myocardial contractility and heart rate are also regulated by NO, which inhibits contractile tone, reduces myocardium oxygen consumption and opposes the inotropic and chronotropic actions of catecholamines (Balligand and Cannon 1997). NO is also involved in the cardiac response to ischemia. An appropriate concentration of NO may attenuate ischemic damage to the heart when coronary blood flow is insufficient (Raij 2006); however, post-ischemia myocardial

damage after reperfusion is partially caused by NO-derived reactive nitrogen intermediates, such as peroxynitrite, which can promote post-ischemic myocardial apoptosis and necrosis (Schulz et al. 2004).

At physiological concentrations, NO plays a significant role in the control of renal function and long-term blood pressure regulation. NO can regulate renal hemodynamics by selectively dilating the afferent arterioles in the cortical nephron, thereby increasing the glomerular filtration rate (Adam and Raij 2000). NO also modulates renin secretion by the juxtaglomerular apparatus and sodium and water transportation in various nephron segments (Ortiz and Garvin 2002; Herrera and Garvin 2005). Renal NO deficiency has been shown to participate in the pathogenesis of chronic renal failure (Blum et al. 1998). Furthermore, results of several studies suggest that iNOS-derived NO could be the direct cause of renal tubular injury in acute renal failure (Goligorsky et al. 2002).

In the liver, low physiological levels of NO generated by the hepatic nerve and hepatic vascular endothelium maintain the hepatic vascular tone and portal pressure necessary to ensure adequate liver perfusion (Shah et al. 2004). Optimal hepatic perfusion determines the rate of energy substrate delivery for metabolism in hepatic cells. In addition, normal perfusion can protect the liver against thrombosis, oxidative stress, and inflammation (Shah et al. 2004). Hepatic NO also protects the liver from ischemia-reperfusion injury after liver transplantation (Becker et al. 2009). Some studies suggest that decreased NO production within the hepatic microcirculation may be important in the development of parenchymal tissue damage and the onset of portal hypertension (Pizcueta et al. 1992). Also, it has been proposed that increased NO synthesis is responsible for the development

of hyperdynamic circulation in cirrhosis (Battista et al.1997; Martin et al. 1998). Thus, appropriate NO activity in the liver is important for the maintenance of normal hepatic function.

Although research into the function of NO in adipose tissue started relatively recently, there have been several interesting findings regarding the physiological role of NO in this tissue. NO was first discovered to be an indispensable factor for the biogenesis of thermogenic mitochondria in interscapular brown adipose tissue (Perez-Matute et al. 2009). Later, in a clinical study, NO was shown to be the most important factor in determining preprandial and postprandial blood flow in adipose tissue, suggesting that NO may be an important regulator of the net flow of energy substrates into and out of adipocytes (Ardilouze et al. 2004).

Besides the physiological roles of NO described above for the heart, liver, kidney and adipose tissue, NO has also been shown to act in other organ systems including the nervous system, muscles, skeletal tissue and gastrointestinal system, as summarized in the following table.

Tissue/organ system	**Action affected by NO**	**Reference**
Central nervous system	Maintenance of sufficient cerebral perfusion	Yun et al. 1997
	Involved in synaptic plasticity associated with learning and memory	Duncan and Heales 2005
	Regulation of drinking and eating behaviors	Calapai and Caputi 1996

Peripheral nervous system	Modulation of pain sensation Regulation of neural regeneration	Holthusen and Arndt 1995 Dawson and Dawson 1998
Skeletomuscular system	Influences the activity of osteoclasts in bone resorption and osteoblasts in bone formation Regulation of skeletal muscle excitation-contraction coupling, blood flow, myocyte differentiation and glucose homeostasis	Van't Hof et al. 2004 Reid 1998
Gastrointestinal system	Regulation of blood flow and motility in the gastrointestinal tract Modulation of nutrient absorption and inflammatory responses	Shah et al. 2004 Lanas 2008
Immune system	Defense against pathogens Prevention of tumor pathogenesis Involved in autoimmune processes and chronic degenerative diseases	Bogdan 2001 Liew 2007

Table 1. Biological roles of NO in various organ systems

Nitric oxide signaling pathways

NO signaling pathways can be roughly divided into cGMP-dependent and cGMP-independent pathways. The cGMP-dependent pathway was the first NO signaling pathway identified. This pathway was initially identified by researchers who were studying mechanisms which induce vascular smooth muscle relaxation (Lincoln et al. 2001). The cGMP-dependent pathway is still considered to be the most important pathway in mediating NO function. Besides inducing vascular smooth muscle relaxation, the cGMP-dependent pathways are also involved in synaptic vesicle recycling in neurotransmission (Petrov et al.

2008), platelet granule secretion in platelet activation (Li et al. 2004 b), regulation of ciliary motility in respiratory epithelium (Li et al. 2000), and many other biological processes. NO elevates intracellular cGMP levels through the activation of soluble guanylyl cyclase (sGC), which can further activate a variety of downstream effector proteins (Ignarro and Sessa 2000). These effector proteins include cGMP-dependent protein kinases (PKG), cGMP-regulated phosphodiesterases (PDEs) and cGMP-activated ion channels (Biel et al. 1999). Single or multiple activated effector proteins can induce different downstream effects in diverse biological systems (Pfeifer et al. 1999). cGMP activation by elevated NO-stimulated sGC is a shared step in all the cGMP-dependent pathways; however, the outcomes of activating the various pathways are very different.

NO-mediated signaling pathways can also bypass cGMP. cGMP-independent signaling pathways are relatively recent discoveries that have opened up an entire new area of investigation in NO molecular signaling. NO has direct effects on the function of ion channels, enzymes, and a number of proteins in other signaling pathways. For example, NO has been shown to directly regulate the function of K_{Ca} channels in airway smooth muscle and calcium-dependent potassium channels in vascular smooth muscle (Bolotina et al. 1994; Abderrahmane et al.1998). Moreover, enzyme activity can be directly altered by NO. One good example is that NO induces S-nitrosylation of protein, which can either stimulate the enzyme activity or inhibit it (Martínez-Ruiz and Lamas 2007). In addition, NO regulates the cell cycle by increasing both mRNA and protein expression for p21, a cell cycle regulator. NO increases p21 mRNA transcription and protein expression by activating the p38 MAPK pathway and stabilizing p21 mRNA (Cui et al. 2005). The mechanism through which NO activates the p38 MAPK pathway involves either stimulating a kinase or inhibiting a

phosphatase via the nitrosylation of the enzyme, thereby leading to an increase in the phosphorylation of p38 MAPK (Huwiler and Pfeilschifter 1999).

(II) Overview of the balance of nitric oxide bioavailability

NO bioavailability is maintained at a steady-state by the continuous generation and removal of NO. Since NO can play paradoxical roles at different concentrations, any force breaking this balance will cause the concentration of NO to shift out of its favorable range. Such a shift may lead to a diseased state if it is allowed to persistent (Birkenhäger et al. 2006). NO is derived from two major sources. It is either generated from a reaction catalyzed by nitric oxide synthase (NOS) or released from NO reservoirs (Knowles et al. 1990; Zweier et al. 1999). NO can be inactivated in two ways. It can be permanently inactivated by undergoing an irreversible reaction with another compound or it may undergo a reversible reaction and convert to the NO reservoirs. Pathways involved in the generation, removal and recycling of NO are presented in Figure 1.

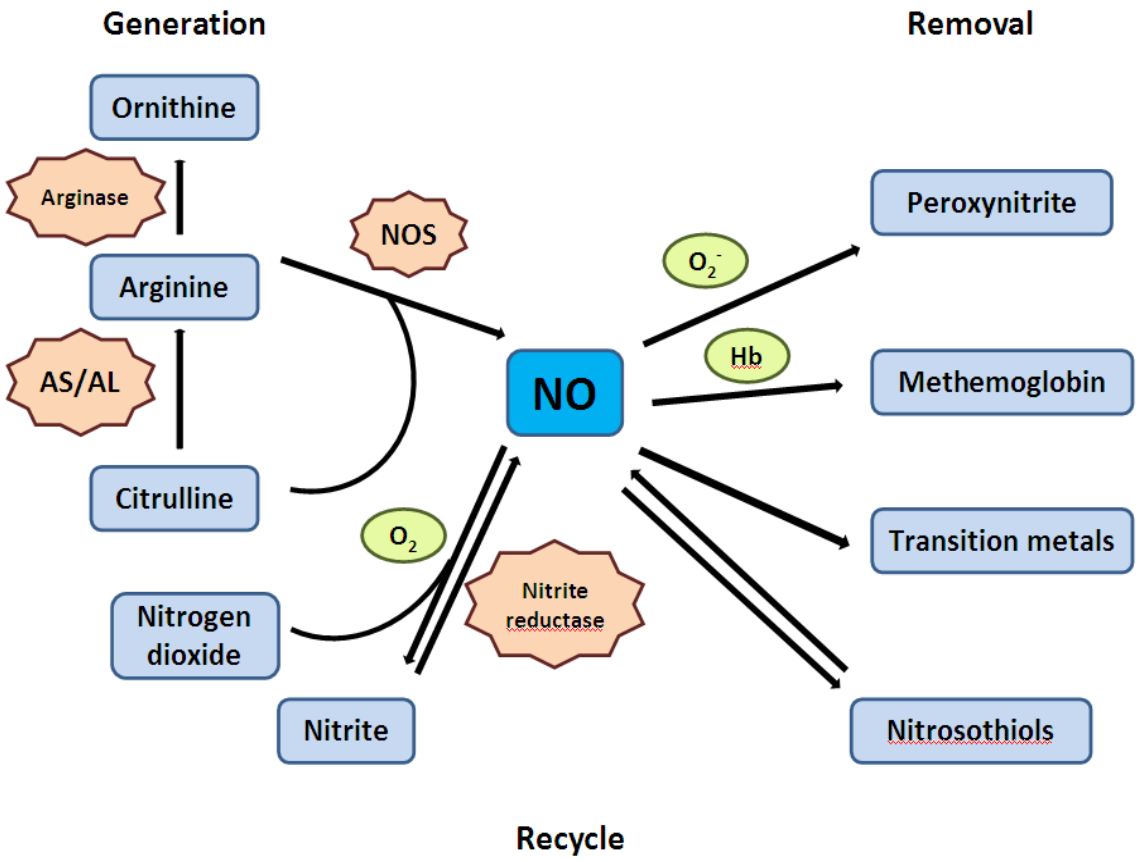

Figure 1. Pathways involved in the generation, removal and recycling of NO

Introduction of nitric oxide synthase isoforms

The reaction that generates NO is catalyzed by a family of enzymes called nitric oxide synthase (NOS). NOS generates NO and L-citrulline from L-arginine and oxygen. The reaction requires several cofactors: nicotinamide adenine dinucleotide phosphate (NADPH), heme, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydrobiopterin (BH4) (Knowles et al. 1990). In mammals, there are three documented NOS isoforms: nNOS (NOS1), iNOS (NOS2) and eNOS (NOS3) (Knowles and Moncada 1994). All three isoforms of NOS are homodimers consisting of two identical monomers

(Sheta et al. 1994). Each monomer is comprised of an N-terminal oxygenase domain and a C-terminal reductase domain as shown in Figure 2 (Alderton et al. 2001).

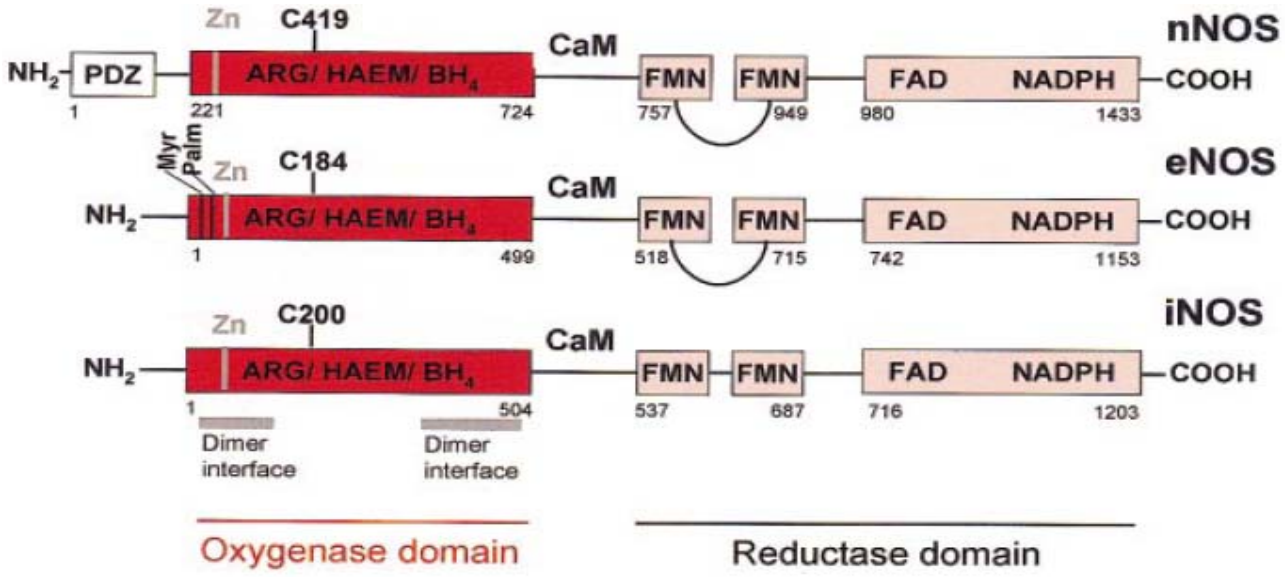

Figure 2. Domain structure of human nNOS, eNOS and iNOS

Taken from: Nitric oxide synthases: structure, function and inhibition. (Alderton et al. 2001)

The oxygenase domain contains cysteine-ligated heme, BH4 cofactor and arginine-binding sites (Ghosh et al. 1997; Zhang et al. 2001). The C-terminal reductase domain has FMN, FAD and NADPH binding sites (Bredt et al. 1991; Alderton et al. 2001). At the junction between these two domains is a calmodulin (CaM) –binding region (Bredt et al. 1991). The homology of the three isoforms is about 50% to 60%, while the homology of a given isoform between species can be as great as 85% to 92%. Although they have considerable structural homology (Charles et al. 1993), these three NOS isoforms differ in molecular weight, intracellular location and factors that regulate the enzymes (Bian et al. 2006).

Endothelial nitric oxide sythase. Endothelial NOS is the most studied member of the NOS family. It is the major NOS isoform expressed in the cardiovascular system under normal conditions. In addition to being expressed in the vascular endothelium, eNOS is also found in blood platelets, cardiac myocytes, hippocampal neurons, epithelial cells in lung and gut and mast cells (Lowenstein and Michel 2006). eNOS is a membrane-associated protein. Until recently, the expression of eNOS was considered to be constitutive; however, new evidence now suggests that eNOS expression can also be induced by some stimulators such as shear stress and estrogen (Chambliss and Shaul 2002 ; Li et al. 2004). The activation of eNOS is Ca^{2+}-CaM dependent. The molecular weight of eNOS is approximately 133 KDa. Several polymorphisms of the eNOS gene have been identified. Studies investigating the influence of polymorphisms on human health, suggest that eNOS gene variants may play a role in development of cardiovascular diseases (Casas et al. 2006).

Neuronal nitric oxide synthase. Neuronal NOS is the largest member of the NOS family with a molecular weight of 160 KDa. Initially, researchers thought nNOS was only present in neuronal tissue where it mediated second messenger signaling across the neuronal synapses (Bredt and Snyder 1994; Cork et al. 1998). More recently, nNOS has been discovered in nonneuronal tissues including skeletal muscle (Kobzik et al. 1994), cardiac muscles (Xu et al. 1999), and the adventitia of a subset of blood vessels at neuronal tissue (Nozaki et al. 1993). The activation of nNOS is also Ca^{2+}-CaM dependent. Even though nNOS expression is considered to be constitutive, its expression pattern is complicated by the presence of several different mRNA splice variants that lead to the generation of four different peptides (Wang et al. 1999). Two of these peptides have special domains that anchor these sub-types to specific sub-cellular structures; the other two peptides are thought to be localized to the cytoplasm

(Mungrue et al. 2003). Because both nNOS gene structure and expression are regulated via a variety of intriguing mechanisms, it is considered as one of the most complex human genes described to date (Wang et al. 1999).

Inducible nitric oxide synthase. Inducible NOS is the smallest member of the NOS family. It was formerly considered to be the inducible NOS isoform until it was found to be constitutively expressed in some epithelial cells in the lung, gastrointestinal tract as well as in a myriad of other cell types (Nathan 1997). iNOS can be induced in almost all tissues, even in those tissues where it is not present under normal condition (Bian et al. 2006). iNOS has several unique characteristics. First, its activity is not dependent upon an increase in the intracellular calcium concentration, because it is tightly and non-covalently bound to calmodulin and calcium at the time it is synthesized (Cho et al. 1992). Second, the primary function of iNOS is to produce a large amount of NO in a short period of time to defend against pathogens (Nathan 1997). It is a very efficient enzyme that can generate several hundred times as much NO as the other two NOS isoforms. Third, iNOS requires *de novo* synthesis in most cells (Charles et al. 1993). Once iNOS is generated, it catalyzes the formation of NO in the cytoplasm (Mungrue et al. 2003).

Nitric oxide synthase regulation

Multiple regulatory pathways are involved in modulating nearly every aspect of NO synthesis and function. These mechanisms control NOS mRNA transcription, posttranslational modification, NOS expression and NOS activity. Even though all three NOS isoforms share some common regulatory pathways, every NOS isoform has its own

distinctive regulatory mechanisms. We can divide these mechanisms into calcium-dependent and calcium-independent mechanisms.

Calcium-dependent mechanism. The activation of all three NOS isoforms requires the binding of a calcium-calmodulin complex to the enzyme; however, the calcium-dependency of this regulation only applies to eNOS and nNOS, not to iNOS. This is because iNOS is already tightly associated with calmodulin at the time of synthesis regardless of intracellular calcium concentration (Cho et al. 1992). The binding of calcium-calmodulin to eNOS and nNOS requires an increase in intracellular calcium concentration to 500 nM or more, which is much higher than the resting calcium level within the cell. So, any compound that can increase the intracellular calcium concentration to this level will cause an increase in NO formation. In endothelial cells, various substances can increase intracellular calcium concentration: bradykinin, histamine, serotonin and many others (Takeda et al. 1992; Linhart et al. 2003). If intracellular calcium levels are chronically elevated, however, there may actually be a decrease in NO formation. Under this condition, NO synthesis is diminished because the Vmax of NOS is decreased due to the phosphorylation of NOS by calcium/calmodulin protein kinase II (Schmidt et al. 1992).

Calcium-independent mechanisms. Calcium-independent regulation of NOS is complex. It includes transcriptional regulation, posttranslational modifications, protein structure modifications and protein-protein interactions (Zhang et al. 2003; Searles 2006). Transcriptional regulation modulates NOS mRNA production. Epigenetic regulation involves the modification of NOS gene activity without changing the underlying DNA sequence. Specific epigenetic processes affecting NOS genes include imprinting, gene silencing, and

DNA methylation (Chan et al. 2005; Matouk and Marsden 2008). Several studies have demonstrated that different NOS genes can be epigenetically regulated in different ways. iNOS genes are regulated predominantly at the transcriptional level (Geller et al. 1993; Xie et al. 1993). For example, hypomethylation of the DNA surrounding the proximal promoter region is a prerequisite for gene activation, whereas heavy methylation leads to gene silencing (Hsieh 1994). The silenced human iNOS promoter has hypermethylation of CpG dinucleotides and histone H3 lysine 9 methylation (Chan et al. 2005). Other studies suggest that histone hyperacetylation inhibits iNOS induction in mesangial cells (Yu et al. 2002; Yu and Kone 2006). Similarly, promoter DNA methylation plays an important role in the cell-specific expression of a constitutively expressed eNOS gene in vascular endothelial cells (Chan et al. 2004). Although eNOS mRNA steady-state levels could be influenced by transcriptional changes, studies indicate that posttranscriptional regulation is more important (Fleming and Busse 2003). The reason posttranscriptional regulation is more important to eNOS may be due to the long eNOS mRNA half-life of 10-35 hours. Thus, stable eNOS mRNA can pool in the cytosol and continuously synthesize proteins even after gene transcription has been turned off (Searles 2006).

Various physiological and pathophysiological stimuli have been shown to modulate the expression of NOS mRNA *in vitro*. These stimuli affect NOS mRNA by modulating mRNA transcription, degradation, transport, localization, and processing. Stimuli believed to increase eNOS expression include transforming growth factor-*b* (TGF-*b*) (Chen et al. 2001), lysophosphatidylcholine (Xing et al. 2008), laminar shear stress in the presence of estrogen (Nishida et al. 1992), hydrogen peroxide (Wartenberg et al. 2003), and oxidized linoleic acid (Ramasamy et al. 1998). Stimuli that decrease eNOS expression include tumor necrosis

factor α (TNF-α) (Lai et al. 2003) and high concentrations of NO; NO inhibits NOS via a negative feedback mechanism (Griscavage et al. 1995). nNOS and eNOS are more sensitive than iNOS to the inhibitory action of NO. iNOS may be insensitive to NO negative feedback in order to generate large quantities of NO for defending against pathogens (Griscavage et al. 1995). There are several stimuli, such as oxidized LDL and hypoxia, that can either increase or decrease eNOS expression depending on LDL level or severity of hypoxia (Searles 2006).

Alterations in NOS cofactors and substrates availability play a critical role in regulating NOS activity. A phenomenon called "NOS uncoupling" refers to a dysfunction of NOS that occurs when cofactors and substrates are inadequate, and the enzymatic reduction of oxygen molecules by NOS is no longer coupled to arginine oxidation. As a consequence, NOS starts to generate superoxide instead of NO (Pou et al. 1992). This phenomenon was first recognized in purified nNOS and later observed with eNOS and iNOS (Channon 2004). Research has demonstrated that decreased heat shock protein (Hsp) 90 recruitment (Pritchard et al. 2001), arginine (Schneider et al. 2003), and tetrahydrobiopterin (Alp and Channon 2004) can lead to NOS uncoupling. In the case of BH_4 deficiency, eNOS will generate superoxide rather than NO (Katusic et al. 2009). The reactive oxygen species (ROS), in turn, degrades BH_4, which further lowers BH_4 levels. This creates a vicious cycle that decreases NO synthesis and increases ROS (Alp and Channon 2004). Similarly, decreased levels of arginine also can downregulate NO and upregulate superoxide production (Mueller et al. 2005).

NOS activity can be altered by modifying the structure of the enzyme by phosphorylation, nitrosylation or acylation. Regulation of eNOS activity through multi-site phosphorylation of

certain serine and threonine residues has been investigated. To date, six sites of phosphorylation have been identified in bovine eNOS at Tyr-83, Ser-116, Thr-497, Ser-617, Ser-635, and Ser-1179, which are equivalent to Tyr-81, Ser-114, Thr-495, Ser-615, Ser-633, and Ser-1177 in the human sequence (Michell et al. 2002; Venema 2002; Li et al. 2007; Fulton et al. 2008). Phosphorylation of different sites can increase, decrease or have no effect on NOS activity. For example, phosphorylation of eNOS-Ser (1177) can fully activate eNOS activity (McCabe et al. 2000); however, NO synthesis is inhibited by phosphorylation of eNOS-Ser (116) (Bauer et al. 2003). eNOS phosphorylation occurs in response to a variety of humoral, mechanical and pharmacological stimuli. The regulation of phosphorylation involves numerous kinases and phosphatases, as well as interactions with other eNOS regulatory mechanisms such as intracellular calcium levels (Dudzinski and Michel 2007), protein-protein interactions and the regulation of subcellular localization (Michell et al. 2002; Mount et al. 2007).

NO can directly modify sulfhydryl or cysteine residues of proteins through **S**-nitrosylation. **S**-nitrosylation is one of the most important protein modifications in signal transduction (Sun et al. 2006). S-nitrosylation of eNOS leads to enzyme inhibition whereas de-nitrosylation is associated with an increase in enzyme activity (Dudzinski and Michel 2007). NO gas and NO donors induce S-nitrosylation on residues Cys 94 and Cys 99 of eNOS and downregulate eNOS activity as a consequence (Erwin et al. 2005). NOS is only active as a homodimer, and cysteine residues in eNOS are particularly important for both dimer stability and enzyme activity (Erwin et al. 2005). A high concentration of NO induces S-nitrosylation of cysteine residues, which causes eNOS to shift from the active dimeric form to the inactive monomeric form of the enzyme (Ravi et al. 2004). This explains the negative feedback mechanism of

NOS regulation by NO. Sub-cellular localization also has an impact on eNOS S-nitrosylation due to the membrane targeting required for S-nitrosylation (Ravi et al. 2004).

Catalytically active eNOS is located on the Golgi complex or in the caveolae of endothelial cells (Liu et al. 1997). Mislocalization of eNOS decreases enzyme activity and impairs NO production. Experimental evidence from one study suggests that the stimulated production of NO is markedly reduced even when the purified mislocalized eNOS is catalytically identical to wild-type (Sessa et al. 1995). Hence, optimal NO synthesis requires correct eNOS targeting, which can be modulated by myristoylation and palmitoylation. Irreversible myristoylation of glycine-2 initially targets eNOS to the cell membrane, where reversible post-translational palmitoylation of the Cys 15 and Cys 26 residues occurs (García-Cardeña et al. 1996). Myristoylation and palmitoylation are subjected to extracullular signals, which provides a mechanism for dynamic regulation of eNOS localization (James and Olson 1989; Degtyarev et al. 1993). Prolonged agonist stimulation of eNOS induces depalmitoylation and mistranslocation, which serves as one of the mechanisms for decreasing NO production by eNOS (Liu et al. 1996).

There are two caveolar proteins that play important roles in NOS regulation. One of these proteins is caveolin, which has a molecular weight of 22 kDa. Caveolin-1 and caveolin-2 are expressed in endothelial cells; caveolin-3 is a muscle-specific isoform that is expressed in cardiomyocytes and skeletal muscle cells (Smart et al. 1999). Under resting conditions, the binding of NOS with caveolin tonically inhibits NOS. Disassociation of NOS and caveolin is required for the full activation of NOS. Another protein named endoglin also plays a critical role in the regulation of eNOS catalytical activity. Endoglin is a 180 kDa glycoprotein that is

highly expressed in the caveolae of endothelial cell membranes (Gougos and Letarte 1990). Endoglin stabilizes eNOS by promoting its association with Hsp90 (Bernabeu et al. 2007). Without endoglin, the half-life of eNOS is diminished and the interaction between eNOS and hsp90 is impaired. Moreover, eNOS uncoupling occurs which results in the generation of superoxide instead of NO (Dudzinski and Michel 2007). Hsp90 is a chaperone protein that is involved in protein trafficking, protein folding and agonist-dependent eNOS activation (Richter and Buchner 2001). eNOS becomes robustly associated with hsp90 as hsp90 undergoes reversible tyrosine phosphorylation. Hsp90 binding stimulates eNOS activity by enhancing the binding affinity of eNOS for calmodulin (Dudzinski and Michel 2007).

iNOS is specifically associated with a protein called kalirin (Ratovitski et al. 1999a). Cells overexpressing kalirin demonstrate decreasing iNOS activity without affecting iNOS expression after stimulation by lipopolysaccharide (LPS) and interferon. It has been suggested that kalirin inhibits iNOS activity by impairing iNOS homodimerization (Zhang et al. 2003). Nitric oxide associated protein 110 (NAP110) is a protein which can form physiological complexes with iNOS to inhibit its activity (Ratovitski et al. 1999b). Similar to kalirin, NAP110 may impair iNOS homodimerization, thereby reducing NO production. Another iNOS associated protein is a GTPase called Rac, which plays an important role in controlling the spatial distribution of iNOS in cells (Zhang et al. 2003). Furthermore, nNOS has a PSD-95/Dlg/ZO-1 (PDZ) domain. Direct interactions of a variety of proteins with PDZ domains of nNOS have been shown to influence the subcellular distribution and/or activity of nNOS in brain, muscle and the macula densa (Kone 2000).

Nitric oxide and reactive oxygen species

ROS are natural byproducts of normal metabolism which play regulatory roles in cellular function. These highly reactive molecules interact irreversibly with proteins (Stadtman 1993), lipids (Gutteridge 1995), and DNA (Feig et al. 1994), as well as with small biological molecules such as NO (Koppenol et al. 1992). ROS can be made from a variety of enzymatic and non-enzymatic sources. Among the large family of ROS, superoxide is the most studied member with respect to NO metabolism (Lubos et al. 2008). Potential sources of vascular superoxide production include NADPH-dependent oxidases (Rajagopalan et al. 1996), xanthine oxidase (White et al. 1996), lipoxygenase, mitochondrial oxidases and NOS synthases (Vasquez-Vivar et al. 1998). NADPH oxidase and the NO synthases appear to have the most important impact on NO bioavailability. The NADPH oxidase enzyme complex catalyzes the one-electron reduction of molecular oxygen using NADPH as an electron donor and the reduced form of cytochrome to generate superoxide (Isogai et al. 1995). NOS uncoupling also can produce superoxide instead of NO as described earlier in this review. Once the highly reactive superoxide is made, it can rapidly react with NO to form peroxynitrite (Radi and Alvarez 2003).

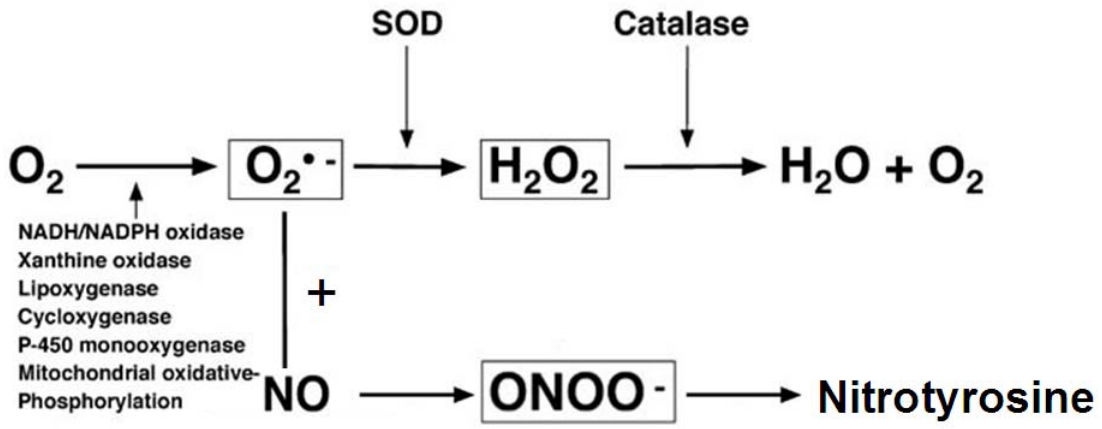

Figure 3. Reaction of NO and superoxide

Based on Figure 1 from "Atheroprotective effects of antioxidants through inhibition of mitogen-activated protein kinases" (Moe et al. 2004).

Peroxynitrite is not only a highly reactive molecule but also a potent oxidant. Toxic peroxynitrite contributes to the pathogenesis of various diseases such as atherosclerosis (Kojda and Harrison 1999), inflammation (Gookin et al. 2005), and neurodegenerative disorders (Torreilles et al. 1999). The majority of peroxynitrite is produced by NO reacting with superoxide. Because nitric oxide can easily traverse the cell membrane, it is able to diffuse into cells and react with superoxide close to the sites of superoxide formation. Peroxynitrite is also able to cross cell membranes by passive diffusion. Because peroxynitrite is highly reactive, it has an extremely short half-life. Peroxynitrite can be inactivated or converted into different substrates by several pathways. First, peroxynitrite reacts with metal- and selenium-containing proteins and other proteins that do not contain prosthetic groups (Radi and Alvarez 2003). Second, peroxynitrite can form hydroxyl and nitrogen dioxide radicals; however, only a small fraction of the

peroxynitrite formed is converted to radicals (Koppenol et al. 1992). Third, low molecular weight antioxidants, particularly glutathione, inactivate a small proportion of peroxynitrite to protect cells from oxidative damage (Torreilles et al. 1999).

Peroxynitrite alters the structure and function of cell membrane proteins by oxidization, nitration and minor nitrosation (Miersch and Mutus 2005). Peroxynitrite and free radicals derived from peroxynitrite can rapidly oxidize sulfur-containing amino acids and aromatic amino acids, such as cysteine, tryptophan and phenylalanine (Radi and Alvarez 2003). It has been shown that peroxynitrite or its derived free radicals can react with twenty different amino acids; however, only cysteine, methionine and tryptophan can directly react with peroxynitrite (Radi and Alvarez 2003). The reaction of peroxynitrite with the thiols of free cysteine and albumin was the first-reported direct reaction with peroxynitrite. Peroxynitrite reacts with the thiol side chain of cysteine to form S-nitrosothiol. It is well known that thiol groups are very important for normal protein function. Inactivation by oxidation of their cysteinyl thiols has been demonstrated to occur in more than 240 enzymes (Wallace 1997; Denu and Tanner 1998). This group of enzymes includes proteins that serve important roles in cellular signaling pathways, such as thiol-containing tyrosine phosphatases, transcription factors and cysteine proteases (Radi et al. 1991). The nucleophilic sulfur atom in the side chain of methionine is also susceptible to oxidation by peroxynitrite (Perrin and Koppenol 2000). The oxidation of methionine by peroxynitrite has been observed in the antitrypsin inhibitor protein and in glutamine synthetase (Ma et al. 1999; Taggart et al. 2000). Tryptophan also reacts with peroxynitrite to form nitrotryptophan, which explains the altered function of tryptophan caused by nitric oxide (Yamakura et al. 2003). Peroxynitrite-dependent tryptophan modification can also be linked to the loss or alternation of protein function (Radi and Alvarez 2003).

As a potent oxidant, peroxynitrite can react with transition metal centers, such as the Mn ion in Mn superoxide dismutase (Quijano et al. 2001) and the Fe in hemoglobin (Mehla et al. 1999). Generally speaking, the reaction of peroxynitrite with transition metals can lead to the formation of secondary oxidizing species and nitrogen dioxide. This is a reversible process. Those oxidizing species may be reduced back in the presence of reductants, such as glutathione or ascorbic acid (Alvarez and Radi 2003). Under certain conditions, the oxidizing species can further react with other molecules to cause impairment. For example, if the oxidizing species is formed with a metal in the active site of an enzyme, it may react with a critical amino acid nearby to induce a loss of enzyme function (Zou et al. 1997). On the other hand, peroxynitrite can also oxidize reduced metal centers by two electrons yielding the oxyradical or oxo-compound accompanied by the formation of nitrite instead of nitrogen dioxide. Hence, the interactions of peroxynitrite with transition metal centers are complex and may ultimately diminish or amplify the oxidative outcome (Radi and Alvarez 2003).

Peroxynitrite-derived secondary radicals, such as hydroxyl, carbonate and nitrogen dioxide radicals, react with other amino acids that do not react directly with peroxynitrite, such as tyrosine (Pietraforte et al. 2003). When tyrosine is exposed to peroxynitrite, it will lead to the formation of 3-nitrotyrosine, 3-hydroxytyrosine and 3,30-dityrosine (Radi et al. 2001); however, the yield of 3-nitrotyrosine formation is less than 10% of the total liberated tyrosine (Hensley et al. 1997). Nitrotyrosine formation could lead to a loss of protein function (Levine and Stadtman 2001). Most importantly, protein nitration may interfere with signal transduction cascades because nitrated tyrosyl residues cannot be phosphorylated (Kong et al. 1996). In essence, protein nitration is interfering with downstream events that require phosphorylation to be activated, as is the case with thiol-containing tyrosine phosphatases, certain transcription factors

and cysteine proteases (Takakura et al. 1999). Histidine is another amino acid that does not react directly with peroxynitrite but can be modified by peroxynitrite-derived secondary radicals (Alvarez et al. 1999). Histidine is a possible candidate for site-specific modification by peroxynitrite because histidine is present as a metal ligand in a number of proteins (Alvarez and Radi 2003). Phenylalanine does not react directly with peroxynitrite. Nevertheless, its exposure to peroxynitrite leads to the formation of p-, m- and o-tyrosine, as well as nitrophenylalanine. The formation of o- and m-tyrosine, nitrophenylalanine and nitrotyrosine are used as indicators of peroxynitrite formation (Alvarez et al. 1999).

Measuring nitrotyrosine as an indicator of the amount of NO scavengered by superoxide is a frequently used technique; however, there are several problems regarding the specificity of this technique. First, other pathways have been proposed for nitrotyrosine formation which bypass NO derived peroxynitrite. Nitration can be mediated by peroxidases such as myeloperoxidase in the presence of hydrogen peroxide and nitrite (Eiserich et al. 1998). In addition, tyrosine nitration can occur from the reaction of the tyrosyl radical with nitric oxide followed by further oxidation to yield nitrotyrosine (Ischiropoulos et al. 1992; Beckmann et al. 1994). In order to attribute nitrotyrosine to peroxynitrite formation, additional evidence may be necessary. For example, if nitration is mediated by myeloperoxidase, another byproduct, chlorotyrosine, should be observed as well. But if nitration is mediated by peroxynitrite, hydroxytyrosine would be expected (Linares et al. 2001). There is no doubt, however, that protein nitration is a hallmark of the contribution of nitric oxide to oxidative damage. The improvement of up-to-date techniques for measuring nitrotyrosine, including the development of specific antibodies, is providing a more specific measurement. As an example, a proteomic approach using a monoclonal antibody

against nitrotyrosine has led to the identification of over 40 different proteins that appear to undergo nitration during inflammatory challenges in vivo (Aulak et al. 2001).

Availability of substrates and cofactors of nitric oxide synthase catalyzed reactions

Arginine, the precursor of NO, can also be used to produce urea, polyamines and proline (Wu and Morris 1998). The availability of intracellular arginine is a rate-limiting factor in NO production (Mori and Gotoh 2004). One source of arginine is citrulline. Arginine is synthesized from citrulline in reactions catalyzed by argininosuccinate synthetase (AS) and argininosuccinate lyase (AL); AS and AL are the third and fourth enzymes in the urea cycle (Wu and Morris 1998). The major site of arginine metabolism is the liver. In the liver, arginine that is generated in the urea cycle is rapidly converted to urea and ornithine by arginase. The second major site of arginine formation is the kidney. Here, arginine is synthesized from citrulline and released into the blood. Many other tissues and cell types also contain low levels of AS and AL (Morris 2004).

In adult animals, citrulline is produced by the small intestine primarily from NH_3, CO_2, and ornithine by carbamylphosphate synthetase I and ornithine transcarbamylase, which are the first two enzymes of the urea cycle (Windmueller and Spaeth 1981). The citrulline generated by the actions of these enzymes is the primary source of precursor for arginine synthesis by the kidney and probably other tissues. Citrulline is also formed from arginine as a byproduct of NOS action (Ruiz et al. 1999). This citrulline may be recycled to arginine if AS and AL are present in the same cell, forming the citrulline-NO cycle. At the same time, arginase hydrolyzes arginine to urea and ornithine. Thus, arginase and NOS compete with each other for arginine because they both use the same substrate (Morris 2004 ; Mori 2007).

Arginine is regulated by the arginase and citrulline-NO cycle (Mori and Gotoh 2004). Arginase appears to inhibit NO production via several possible mechanisms. First, arginase decreases intracellular arginine concentration by converting it to urea and ornithine. Second, arginase can induce NOS uncoupling, which could result in the generation of the NO scavenger, superoxide (Chicoine et al. 2004). Third, the overexpression of arginase represses the translation and stability of inducible NOS protein, inhibits inducible NOS activity via the generation of urea, and sensitizes NOS to the endogenous NOS antagonist dimethyl-L-arginine (Durante et al. 2007). The two key enzymes in the citrulline-NO cycle, AS and AL, also play roles in regulating NO synthesis. iNOS and AS are coinduced in immunostimulated murine macrophage-like cells (Nussler et al. 1994), cultured rat aortic smooth muscle cells (Hattori et al. 1994), and cultured rat and human pancreatic cells (Flodstrom et al. 1995). AL was not induced in these cells, perhaps because only AS is a rate-limiting enzyme in the citrulline-NO recycling reaction. Arginine itself can regulate AL and AS in a concentration-dependent manner. The activities of AL and AS are repressed by arginine and increased when arginine is replaced by citrulline in several nonhepatic cell lines (Morris 2004).

Other nitric oxide recycling pathways

There are several pathways for recycling NO so that it can be available in a biological pool rather than being lost. NO can either temporarily bind to some substrates to stabilize it or be reversibly converted to another chemical compound with a longer half-life. A good example is when NO reacts with thiol groups of amino acids, peptides or proteins to form nitrosothiols (Zhang et al. 2004), which are currently considered to be the carriers and reservoirs of NO (Ng and Kubes 2003). After it was discovered that NO reacts with thiols in proteins to form *S*-nitrosothiols

(SNOs), Ng et al. reported the presence of a circulating pool of *S*-nitrosoalbumin in plasma whose levels were coupled to NOS activity. Further research showed that S-nitrosothiols have a plasma concentration that is 3-4 orders of magnitude higher and half-lives that are much longer than free NO, which make them ideal reservoirs and effective buffers for NO (Stamler et al. 1992). Also, SNOs are resistant to inactivation by ROS (Morris 2004). They circulate in plasma primarily as S-nitrosoproteins. The predominant species is *S*-nitrosoalbumin which accounts for 80% of total S-nitrosoproteins (Stamler et al. 1992). Albumin has both a hydrophobic pocket and bound metals that can facilitate *S*-nitrosylation by NO. On the other hand, hemoglobin has several channels through which it can react with NO or S-nitrosoglutathione (GSNO) to produce SNO-hemoglobin (Gow 1998). Abundant evidence also suggests that GSNO can serve as a NO transporter and reservoir in the circulation (Rassaf et al. 2002). S-nitrosylation is a reversible reaction which may be governed by O_2 tension, pH, and other factors associated with hypoxia (Doctor et al. 2005); however, details of the mechanism are still controversial. Numerous laboratories have verified that SNO-albumin, GSNO, and SNO-hemoglobin are present not only in circulation but also in various tissues (Morris 2004).

Besides reacting with NO to form nitrosohemoglobins, hemoglobin can reversibly or irreversibly inactivate NO by other reactions. Researchers have shown that hemoglobin not only serves as a NO transporter but also acts as a NO destroyer (Kim-Shapiro et al. 2006). Hemoglobin inactivates NO by means of two major reactions. NO reacts with oxygenated hemoglobin to form methemoglobin and nitrate. This reaction occurs at an extremely rapid rate and is irreversible. Hence, plasma NO can be efficiently scavenged by this mechanism. Inactivation by oxygenated hemoglobin is considered to be a NO consuming reaction under physiological conditions (Joshi et al. 2002). NO also reacts with deoxygenated hemes to form iron-nitrosohemoglobin (Kim-

Shapiro et al. 2006; Isbell et al. 2008). The role of nitrosohemoglobin in NO regulation has not been fully explained and is still under investigation.

Results of recent research suggest the presence of another NO recycling pathway, called the nitrate-nitrite-nitric oxide pathway. Nitrite in the blood comes from several sources. NO reacts with oxygen and yet-to-be identified NO oxidases to produce nitrite (Kim-Shapiro et al. 2006). Also, some nitrite comes from dietary intake, and some nitrite is formed from nitrate by bacteria residing in the oral cavity (Doel et al. 2004). Most of the plasma nitrate comes from a reaction between NO and oxygenated hemoglobin. Recent evidence suggests that nitrite plays multiple roles in the vasculature. Plasma and tissue nitrite serves as a bioavailable storage pool of NO that is influenced by the physiological oxygen gradient that regulates vascular activity (Li et al. 2004 a). Nitrite also plays an important role in hypoxic vasodilatation (Crawford et al. 2006). *In vivo,* nitrite may be converted to NO by several mechanisms, including the direct reduction of nitrite to NO under acidotic and highly reduced conditions by a process called acidic disproportionation (Zweier et al. 1995). In addition, nitrite is enzymatically converted to NO by xanthine oxidoreductase using deoxyhemoglobin and deoxymyoglobin as substrates (Millar et al. 1998; Cosby et al. 2003; Lundberg et al. 2008).

(III) Regulation of nitric oxide bioavailability by diet

Dietary factors can modulate NO production. Macronutrients including proteins, carbohydrates and saturated and unsaturated fatty acids have different effects on NO synthesis. In addition, a mix of nutrients may have a more complicated influence on NO

generation compared to the effects of a single dietary constituent. Even a single dietary factor may regulate NOS isoforms differently or have the opposite effect on NO production under different conditions. A more thorough knowledge of dietary contributions to the maintenance of optimal NO bioavailability will lead to a better understanding of the significance of dietary control over NO production as it relates to disease.

Regulation of nitric oxide production by proteins. There are a number of dietary proteins that have an impact on NO production. Casein is a phosphoprotein which accounts for nearly 80% of the protein in milk or cheese. Feeding young rats insufficient casein significantly decreases NO produced by constitutive NOS (Wu et al. 1999). In another study, feeding adult rats a protein-deficient diet distinctly decreased systemic NO, renal NO and maximally induced NO synthesis compared to rats fed a protein rich-diet (Tolins et al. 1995; Wu et al. 1999). This research suggests that NO synthesis is impaired in protein-deficient animals. Among all the amino acids in the diet, arginine plays the most critical role in NO synthesis because it is the amino acid used to make NO. Increasing the extracellular concentration of arginine or feeding experimental animals an arginine rich-diet increases NO production from both constitutive NOS and inducible NOS (Wu et al. 1999). Moreover, increasing the extracellular concentration of arginine *in vitro* increases NO production in a dose-dependent manner by many cells, including endothelial cells (Arnal et al. 1995) and activated macrophages (Kepka-Lenhart et al. 2000). The consumption of a 1% arginine diet by young rats increases both the plasma arginine concentration and NO production compared to rats consuming an arginine-free diet (Wu et al. 1999). Thus, sufficient arginine intake helps to maintain optimal NO availability in circulation.

Citrulline is the precursor for arginine synthesis. Increasing the extracellular concentration of citrulline supports NO synthesis in many cell types by increasing the concentration of arginine (Wu and Morris 1998). Glutamine plays a more complex role than arginine. As an important regulator of endothelial NO synthesis, glutamine markedly decreases endothelial NO production in experiments using cultured cells and intact blood vessels from rats (Hecker et al. 1990; Swierkosz et al. 1990). In contrast, glutamine increases the production of NO generated by nNOS via a process mediated by NMDA receptors and the opening of Ca^{2+} channels (Atlante et al. 2001). Glutamine also increases inducible NO synthesis in response to LPS and cytokines in activated macrophages (Bellows and Bernard 1999). Thus glutamine inhibits NO production in the endothelium while increasing NO levels in the nervous system. Lysine is another amino acid that inhibits NO production. Because lysine competes with arginine for the same transport system to enter the cells, it directly reduces the intracellular concentration of arginine (Hucks et al. 2000). Homocysteine has been shown to impair endothelium-dependent relaxation by decreasing NO synthesis in both endothelial cells and platelets (Emsley et al. 1999). Since homocysteine is synthesized from methionine, dietary methionine overload also inhibits NO production (Mutus et al. 2001). Finally, taurine inhibits iNOS expression and inducible NO synthesis in various cell types, such as hepatocytes, macrophages and glial cells (Redmond et al. 1996; Liu et al. 1998; Barua et al. 2001).

Regulation of nitric oxide production by carbohydrates. NO synthesis requires NADPH, which is provided when glucose is metabolized via the pentose phosphate pathway (Wood 1986). Thus, cellular NO synthesis is glucose-dependent. Insufficient glucose intake can suppress NO synthesis in many cells (Wu et al. 2001); however, high

concentrations of glucose will also inhibit NO production (Chu and Bohlen 2004). Plasma glucose concentrations from 5 to 30 mM induce a dose-dependent decrease NO production *in vitro* (Trachtman et al. 1997). Studies with diabetic patients and experimental animals have shown that hyperglycemia impairs NO synthesis by the endothelium (Chakravarthy et al. 1998; Williams et al. 1998). Even though the mechanisms are still not very clear, the most popular hypothesis is that glucose metabolites, glucosamine or advanced glycosylation end products inhibit NO synthesis by endothelial cells (Chakravarthy et al. 1998; Wu and Morris 1998). The long term consumption of a high fructose diet has been shown to cause hypertension and insulin resistance in certain animal models; however, no mechanism showing how high fructose can inhibit eNOS activity has been proposed (Takagawa et al. 2001). In addition, experimental evidence shows that elevated concentrations of glucose or fructose inhibit NO production from iNOS (Tseng et al. 1997).

Regulation of nitric oxide production by fats. Fat is one of the most studied dietary components with respect to the regulation of NO synthesis. The typical Western diet is a moderately high fat diet in which 32% of the total calories are from fat (U.S. Department of Agriculture 2004). The long-term consumption of a high fat diet decreases plasma and urine stable NO metabolites in human subjects or animal models. Dobrian et al (2001) used a moderately high fat diet (32%) to investigate the effect of diet-induced obesity on the development of hypertension. In their studies, half of the rats consuming the moderately high fat diet for 10-16 weeks developed hypertension. The hypertensive rats had significantly lower levels of urine and plasma NO metabolites than the normotensive rats. Several studies have shown that female rats fed a high-fat, refined carbohydrate diet for 7 months to 2 years had reduced plasma or urine levels of stable NO metabolites (Roberts et

al. 2000, 2003, 2005). Later, several studies discovered that the short-term consumption of high fat diet or even a high fat meal can impair endothelial function (Fard et al. 2000; Bourgoin et al. 2008; Kim et al. 2008). All of these studies suggest that dietary fat plays a critical role in the regulation of NO production.

Excessive consumption of saturated fat can cause hypercholesterolemia and high plasma levels of low-density lipoprotein (Brown and Hu 2001). Although definitive mechanisms responsible for the effects of excessive dietary fat on NO production are still not clear, several hypotheses have been proposed. First, high fat intake can upregulate caveolin expression. This may enhance the inhibitory effect of caveolae on NOS and reduce NO production (Yang et al. 2007). Second, the excessive ingestion of lipids increases ROS in circulation and in many organs (Carlsson et al. 1999; Kunsch and Medford 1999; Lopes et al. 2003). ROS not only scavenge NO but also interfere with NOS mRNA transcription by modifying transcription factors, such as nuclear factor-kappaB (NF-kappaB) (Ilan et al. 2005).

Ingesting large amounts of unsaturated fatty acids may have the opposite effect on endothelium-derived NO synthesis as the ingestion of saturated fatty acids. Experimental evidence has shown that most polyunsaturated fatty acids upregulate NO synthesis in endothelial cells (López et al. 2004). Examples of polyunsaturated acids that have been shown to induce endothelium-dependent relaxation via increasing NO synthesis are eicosapentaenoic acid and docosahexaenoic acid (Lawson et al. 1991; Okuda et al. 1997). Eicosapentaenoic acid can increase the intracellular calcium concentration and cellular translocation of eNOS (Omura et al. 2001). In a human study, it was shown that patients

fed a diet supplemented with fish oil which contains eicosapentaenoic acid and docosahexaenoic acid had a markedly increased urinary levels of stable NO metabolites (Harris et al. 1997). By contrast, another family of unsaturated fatty acids, ω-9 unsaturated fatty acids such as oleic acid, have the opposite effect on the regulation of NO synthesis. Oleic acid may inhibit endothelial NO synthesis by suppressing eNOS activity via a protein kinase C-independent mechanism (Davda et al. 1995). Interestingly, ω-3 and ω-6 polyunsaturated fatty acids inhibit inducible NO synthesis by inhibiting iNOS transcription (Ohata et al. 1997; Khair-Eldin et al. 2001), whereas ceramide and linoleic acid enhance iNOS expression and subsequently NO synthesis (Darmani et al. 1995). Besides the issue of saturated fat vs unsaturated fat (Song et al. 2006), a lot of other factors have to be taken into consideration when investigating the role of dietary fat in the regulation of NO production, such as genetic sensitivity (Dobrian et al. 2001) and the gender of the research subjects (Kauser and Rubanyi 1993).

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Chapter II

The short-term consumption of a moderately high fat diet alters nitric oxide bioavailability in female lean Zucker rats

Abstract

Decreased nitric oxide (NO) production associated with the chronic inhibition of endothelial nitric oxide synthase (eNOS) plays an important role in the pathogenesis of various diseases. Researchers have shown that NO production is diminished in rats maintained on high fat diets for extended periods of time, suggesting that eNOS may be affected by increased fat consumption. The objectives of the present study were to determine whether the effects of a moderately high fat diet (MHFD) on NO production are time-dependent and to determine whether eNOS expression in certain tissues is affected by the short-term consumption of a MHFD. To accomplish these objectives, 64 lean Zucker rats were randomly divided into two groups. One group was fed standard rat chow in which 13% of the total calories came from fat. The other group was fed a MHFD in which 32 % of the total calories came from fat for 4 weeks. Nitric oxide production was estimated weekly by measuring the excretion of stable nitric oxide metabolites (NOx) in the urine. Eight rats from each group were randomly selected and sacrificed at weekly intervals. Heart, liver, kidney medulla, kidney cortex and adipose tissue were harvested, frozen in liquid nitrogen and stored at -80^{o}C until they were processed for analysis. Plasma was collected for NOx determination. Compared to rats eating standard

rat chow, rats on the MHFD exhibited a significant decrease in hepatic eNOS expression within the first week. By week 3, there was a significant decrease in cardiac eNOS expression and by week 4, eNOS expression in the kidney medulla had also declined. By contrast, there was no change in eNOS expression in the kidney cortex or adipose tissue for the duration of the study. Plasma NOx levels and urinary excretion of NOx were significantly lower in rats fed the MHFD for the 4 week duration of the study. Because of the early response of the liver to the MHFD, hepatic NOS activity and tissue levels of nitrotyrosine were also determined at week 1. Liver NOS activity was reduced and nitrotyrosine formation was dramatically elevated at this time. By week 4, nitrotyrosine formation in both the liver and kidney medulla in MHFD-fed rats was significantly increased compared to rats fed the control diet. These results indicate that the short-term consumption of a MHFD alters NOx formation and eNOS expression. The effects are tissue specific and time-dependent. The dramatic increase in nitrotyrosine formation suggests that much of the apparent decrease in NOx formation by the liver may actually be due to an increase in the formation of reactive oxygen species (ROS). ROS react with NO to produce peroxynitrite which, in turn, reacts with tyrosine residues in tissue protein to form nitrotyrosine.

Introduction

Nitric oxide (NO) is a small hydrophobic signaling molecule that is synthesized from arginine by nitric oxide synthase (NOS), an enzyme that exists in three distinct isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Alderton et al. 2001). NO signaling plays a central role in the regulation of numerous physiological processes. Because it affects functions within almost every organ system, alterations in NO formation and/or bioavailability have been implicated in the pathogenesis of and complications associated with a wide range of diseases including hypertension (Bian et al. 2008), atherosclerosis (Yang and Ming 2006), erectile dysfunction (Burnett 2006), chronic kidney disease (Baylis 2008), diabetes mellitus (Creager et al. 2003) and Alzheimer's disease (Zhu et al. 2007).

A number of studies have shown that NO production and bioavailability are altered when rodents are fed high-fat diets to induce insulin resistance, obesity and various obesity-associated diseases such as hypertension. In a series of studies where rats were fed a high-fat refined-carbohydrate diet for an extensive period of time, tissue nitrotyrosine levels were elevated after two months, which suggests a decrease in NO bioavailability (Roberts et al. 2000). In addition, endothelial dysfunction and a decrease in NOS protein expression were observed after seven months (Roberts et al. 2005), and the urinary excretion of stable nitric oxide metabolites (NOx) was significantly reduced after 2 years (Roberts et al. 2003). In a study where a moderately high-fat diet was used to induce obesity and obesity-associated diseases in Sprague Dawley rats that were "obesity prone," the urinary excretion of NOx was suppressed at week ten in the rats that became

obese (Dobrian et al. 2003). Similarly, when genetically obese rats and their lean littermates were fed the same moderately high-fat diet, supplemented with 1 % NaCl in the drinking water, there was a significant drop in NOx urinary excretion by week ten in both lean and fat rats on the high-fat, salt-supplemented diet relative to lean and obese rats on the salt-supplemented control diet (Morrison et al. 2007). In all of these studies, NO production and bioavailability were assessed after there was some overt manifestation of insulin resistance or an obesity-associated condition such as salt-sensitivity or hypertension. In the present study, we addressed the question of whether a moderately high-fat diet alone alters NO production and bioavailability and eNOS expression when consumed over a relatively short period of time. Our results show that changes in these parameters occurred within a week and were clearly tissue specific and time dependent.

Methods

***Animals*:** Seven-week-old female lean Zucker rats were purchased from Charles River Laboratories (Wilmington, MA, USA). They were housed, two rats per cage, in plastic cages with corn chip bedding in a room with a 12 hour light/dark cycle and an ambient temperature of 23±2˚C. The rats were allowed to acclimate in the Marshall University Animal Resources Facility for one week before starting the dietary treatment. All procedures using animals were reviewed and approved by the Marshall University Institutional Animal Care and Use Committee.

Research design for the time-course study: Sixty-four rats were randomly divided into 2 groups. One group was fed a standard balanced rodent diet (Rodent Diet 5001, Lab Diet, Brentwood, MO) and the other was fed a moderately high fat diet (formula D12266B, Research Diets, New Brunswick, NJ). The macronutrient composition of these two diets are presented in Table 1. Rats had free access to food and water for the duration of the experiment. Baseline measurements of body weight, water and food consumption, and urine content of stable nitric oxide metabolites (NOx) were obtained before the start of the experiment (week 0). At weekly intervals thereafter, 8 rats from each group were randomly selected and placed in stainless steel metabolic cages to collect a 24-hour urine sample and measure urine output and food and water consumption. Rats were given 24 hours to adapt to the metabolic cages before these measurements were taken. Subsequently, the rats were deeply anesthetized with an intraperitoneal injection of ketamine HCl: xylazine (45: 5 mg/kg) and then exsanguinated by cardiac puncture with a heparinized syringe. The blood was centrifuged and aliquots of plasma were collected immediately and stored at 4˚C and -80˚C for future analysis. A portion of the ventricular wall, the kidneys, and samples of liver and retroperitoneal adipose tissue were harvested and immediately placed in liquid nitrogen. The renal medulla and cortex were separated before the kidneys were frozen. Tissue samples were stored at -80˚C until they could be analyzed.

Western Blot Analysis: Tissues were homogenized with glass homogenizers in ice-cold RIPA buffer (Thermo Scientific, Waltham, MA) containing a mixture of protease inhibitors (Complete Mini, Roche Diagnostics, Mannheim, Germany). Homogenates

were centrifuged at 12,000 g for 10 min at 4˚C and the resultant supernatants were used for Western blot analysis. Protein concentrations were determined using a Thermo Scientific NanoDrop Spectrophotometer (ND-1000). Samples containing equal amounts of protein were separated on 10% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes using a Bio-Rad Criterion Blotter. The membranes were blocked overnight at 4^{o}C in PBS (pH 7.4) containing 5% nonfat dried milk then stained with ponceau S. After they were washed 3 times with PBS, membranes were probed with monoclonal anti-eNOS antibody (Transduction Laboratories, Lexington, KY 1:2000) and anti-GAPDH antibody (Ambion, Austin, TX 1: 10,000) for 1 hour at room temperature. The membranes were washed 3 times with PBS-Tween (PBST) and then incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (Sigma, St. Louis, MO 1: 6000) for 1 hour at room temperature. Afterward, the membranes were washed 3 more times with PBST. To detect GAPDH, membranes were incubated with ECL regular Western Blotting Detection Kit (Amersham life Science, Buckinghamshire, UK) and exposed to films (Thermo Scientific, Rockford, IL). After the membranes were washed 3 more times with PBS, they were incubated with reagents from the ECL Advance Western Blotting Detection Kit to detect eNOS (Amersham life Science, Buckinghamshire, UK) and exposed to films again. Films were scanned using an HP scanjet 4890 and densitomeric analysis was performed using Image J software. To normalize the data, the relative density of each band was divided by the relative density of the GAPDH band in the same lane.

NOx assay: Urine, plasma and tissue levels of nitrate plus nitrite (NOx) were measured using a commercially available kit (NO quantitation kit, Active Motif, Carlsbad, CA). Prior to performing the assay, tissue samples were homogenized in the homogenization buffer used for Western Blotting analysis. Following centrifugation at 12,500×g for 20 minutes, the supernatants were filtered through a 10,000 Dalton micropore Centricon filter (Millipore, Bedford, MA). Plasma samples were also filtered prior to being assayed. Absorbance was measured at a wavelength of 540 nm on a plate reader (Thermo Labsystems, Vantaa, Finland) controlled by Ascent Software. The results for tissue NOx were normalized to the protein concentration of the homogenate.

NOS activity assay: A commercially available kit (NOS Activity Assay Kit, Cayman, Ann Arbor, MI) was used to determine liver NOS activity. Tissues were homogenized in ice-cold homogenization buffer from the kit. The homogenates were centrifuged for 15 minute at 10,000×g at 4 ^{o}C to remove cellular debris. The supernatants were used for the assay, which is based on the conversion of L-[^{14}C] arginine to [^{14}C] citrulline. Upon completion of the assay, the [^{14}C] citrulline in each sample was separated from the L-[^{14}C] arginine by means of an equilibrated resin column. The eluant containing [^{14}C] citrulline was collected in scintillation vials and measured by liquid scintillation spectrophotometry. All samples were measured in duplicate.

Nitrotyrosine ELISA: The nitrotyrosine content of tissues was measured using an immunoassay kit (Oxisresearch, Foster City, CA or Hycult Biotechnology, Netherlands).

Tissues were homogenized in ice-cold RIPA buffer containing protease inhibitors as described for Western Blotting. The homogenates were centrifuged at 12,000×g for 10 min at 4˚C to remove cellular debris. The nitrotyrosine content of the supernatant was determined according to the manufacturer's instructions.

Statistics: Data are expressed as means ± SEM. Statistical differences among means for body weight, food consumption, urine and plasma NOx, the NOS activity assay and the nitrotyrosine ELISA were determined by two-way ANOVA followed by a Tukey test. Western blot data is presented as a percent of the control value for each tissue at the indicated week. For the Western blot data, statistical differences between means were determined by t-tests. Differences were determined to be statistically significant at $P \leq 0.05$. Statistical analysis was performed using SigmaStat software (Jandel Scientific Software, San Rafael, CA).

Results

Weight gain and caloric intake: Although all the rats used in this study gained weight steadily throughout the 4-week treatment period, weight gain by rats on the moderately high fat diet (MHFD) was significantly greater than weight gain by rats on the control diet. The difference between the two groups was significant at week one and remained so for the duration of the treatment period (Figure 1A). There was no difference in caloric intake by the two groups (Figure 1B).

NOx production: Daily NO production was estimated by determining the total quantity of stable NO metabolites (NOx) excreted in the urine over a 24-hour period. NOx

excretion by rats eating the control diet was relatively constant before and during the experimental period. By contrast, NO excretion decreased dramatically when rats were placed on the MHFD (Figure 2). In this group, NOx excretion was reduced over 70% by the end of the first week and remained significantly depressed for the duration of the study. A similar pattern was observed when circulating NOx levels were measured. Plasma NOx levels were essentially unchanged from week to week for rats on the control diet, whereas plasma levels of NOx for rats on the MHFD were significantly decreased at week one and remained so for the rest of the study (Figure 3).

eNOS Western blotting: One mechanism that could be involved in the reduced production of NOx by rats consuming the MHFD is a decrease in the expression of nitric oxide synthase (NOS). We examined this possibility by measuring the expression of one of the NOS isoforms, endothelial NOS (eNOS), by Western blot analysis in various tissues from rats fed control and MHFD for different time intervals. In the kidney, eNOS expression was assessed in both the medulla and the cortex because a preliminary study indicated that there was a noticeable difference in eNOS expression in these two regions of the kidney. Figure 4A shows that the level of expression of eNOS in the medulla is more than 3.6 times greater than the expression of eNOS in the cortex. The bar graphs in figure 4B compare eNOS expression in various tissues from rats fed the control diet and MHFD over a period of 4 weeks. By week 1, there was a marked decrease in eNOS expression in livers from rats on the MHFD compared to eNOS expression in the livers of rats on the control diet. This decrease persisted for the entire 4 weeks of the study. By contrast, the MHFD had no effect on eNOS expression in the other tissues during the first

two weeks of this study. At weeks 3 and 4, eNOS expression by cardiac tissue from rats on the MHFD was significantly lower than eNOS expression in cardiac tissue from rats on the control diet. By week 4, eNOS expression in the renal medulla of rats on the MHFD was also depressed. There was no change in eNOS expression in the kidney cortex during the entire study nor was there a diet-induced change in eNOS expression by adipose tissue.

***NOS activity*:** Of the tissues examined, only liver showed a diet-induced decrease in eNOS expression that paralleled the decline in circulating NOx levels. Therefore we further analyzed liver homogenates for NOS activity to determine whether there was a decrease in NOS activity that corresponded to the decrease in hepatic eNOS expression resulting from the consumption of a MHFD. Results of this assay demonstrate that NOS activity was significantly lower in liver homogenates from rats on the MHFD than in homogenates from rats on the control diet by week 1 (Figure 5). NOS activity remained significantly depressed for the duration of the study; however, the magnitude of the decrease in activity was not as great as the change in eNOS expression. Moreover, the decrease in NOS activity followed a somewhat different pattern than the changes in hepatic NOx levels. Liver NOx levels started to fall at week one but were only significantly depressed by week 2 (Figure 6). Liver NOx levels continued to decline through week 4. Collectively, these results suggest that the decline in eNOS expression does not fully explain the changes in liver NOx production.

***Nitrotyrosine formation*:** An alternative hypothesis to explain the decreased production of NO metabolites by rats on the MHFD is that newly formed NO may be reacting with

reactive oxygen species (ROS) in tissues to produce peroxynitrite, a compound that can further react with the phenolic ring of tyrosine to produce nitrotyrosine. This hypothesis was tested by measuring nitrotyrosine formation in liver from rats on the MHFD and control diet and using nitrotyrosine formation as an indirect measure of ROS production. As shown in Figure 7, the formation of nitrotyrosine by livers of rats on the MHFD increased dramatically compared to nitrotyrosine formation by livers of rats on the control diet. At week 1, more than 3 times as much nitrotyrosine was being formed in livers of rats on the MHFD than in livers of rats on the control diet. Differences were significant at all time points measured. Subsequently, nitrotyrosine levels were measured for other tissues at the time points where they all showed a significant diet-induced decrease in eNOS expression. As shown in Figure 8, there was a significant increase in nitrotyrosine formation in the renal medulla at week 4 whereas there was no diet-induced increase in nitrotyrosine formation in the heart. Figure 8 also shows that significantly more nitrotyrosine is formed per unit of protein in the liver than in the heart and kidney medulla.

Discussion

More and more studies are showing that the chronic consumption of diets high in fat content has serious long-term health consequences (Astrup et al. 2008). One physiological parameter that appears to be seriously compromised by long-term fat consumption is the availability of nitric oxide as indicated by studies showing dramatic declines in the concentration of stable NO metabolites in plasma and urine (Roberts et al.

2003), altered NOS expression (Roberts et al. 2005), and an increased production of NO scavengers (Saiki et al. 2007) in rats maintained on high fat diets over an extended period of time. Furthermore, it has been shown that the pathological changes induced by elevated dietary fat are attenuated by the supplementation of the diet with arginine, a NO precursor (Jobgen et al. 2009a, b). The acute effects of dietary fat on NO bioavailability, by comparison, are still unclear. Therefore, in the present study, we focused on the consequences of short-term consumption of a moderately high-fat diet (MHFD) on systemic NO bioavailability.

The MHFD used in the study presented here is similar in total fat content to that of the typical U.S. diet (U.S.Department of Agriculture 2006). This diet is commercially available and has been used to induce obesity and obesity-associated hypertension in "obesity-prone" Sprague Dawley rats (Dobrian et al 2000; Boustany et al, 2004, 2007). Using the same diet, our laboratory showed that both lean and obese Zucker rats on the MHFD gained significantly more weight over a 10-week period than lean and obese rats fed the control diet (Morrison et al. 2007). In the present study, 7-week-old rats were used and weight gain was monitored at weekly intervals. By the end of the first week, rats on the MHFD had already gained significantly more weight than rats on the control diet. When the experiment was repeated using 7-week-old female Sprague Dawley rats, a similar pattern of weight gain was observed (data not shown). In contrast, when 20- to 22-week-old female hood Wistar rats were fed a similar diet in which 36% of the energy was derived from fat, there was no significant difference in weight gain between rats fed

the high fat diet and their paired controls during the first 4 weeks; however, a significant difference in weight gain was detected by week 14 (Kim et al. 2008). This discrepancy between the two studies may be due to differences in the ages and/or strains of the animals used in the respective studies.

Several studies assessing the impact of dietary constituents on cardiovascular disease have observed that the excretion of stable NO metabolites (NOx) decreases when diets high in fat content are consumed over extended periods of time (Roberts et al. 2000, 2003). Similarly, when investigating the effects of a moderately high fat diet on blood pressure in lean and obese Zucker rats, we observed that the consumption of the MHFD for a period of six weeks also caused a decrease in the urinary excretion of stable NO metabolites (NOx), with the effect being much greater in obese rats than in lean rats (Morrison et al. 2007). In the present study, we focused on time-dependent changes in the systemic NO production in lean rats which we assessed indirectly by monitoring urinary excretion of NOx at weekly intervals (Böger et al. 1996). We observed a statistically significant reduction in NOx excretion within the first week of dietary treatment. In a subsequent experiment, NOx excretion was monitored daily and the difference in NOx excretion was statistically significant at 48 hours (data not shown). When rats on the control diet and the MHFD were sacrificed at weekly intervals, plasma NOx levels were determined. Changes in plasma NOx levels paralleled changes in NOx excretion and were significantly lower in the MHFD group after one week of dietary treatment.

Since plasma NOx levels are thought to generally reflect NO produced by eNOS (Kleinbongard et al. 2003), the expression of eNOS was determined by Western Blot analysis in several different tissues. The most dramatic effects were observed in the liver. Within a week of the start of dietary treatment, there was a marked decrease in liver eNOS expression. This could be due to a direct effect of triglycerides on hepatocyte function as suggested by the work of Kim et al. (2002) who exposed isolated rat liver cells to increasing concentrations of a triglyceride emulsion in culture. They observed a decrease in mRNA and protein expression for both eNOS and iNOS. In the present study, eNOS expression was also suppressed in the heart and kidney medulla at weeks three and four respectively whereas there was no detectable change in eNOS expression in the kidney cortex or adipose tissue. These results suggest that the expression of eNOS in various tissues have different sensitivities to the inhibitory effects of the high fat diet.

To assess pathways involved in the hepatic production and removal of NO, the following parameters were measured: hepatic levels of NOx, NOS activity and nitrotyrosine formation. Although there was a sizeable, time-dependent decrease in hepatic NOx levels, there was only a modest decrease in NO synthase activity, suggesting that other factors are involved in the MHFD-induced decrease in hepatic NOx. To test the hypothesis that the MHFD increases the formation of superoxides which scavenge NO, hepatic nitrotyrosine formation was measured. Nitrotyrosine formation is an indirect measure of the amount of NO scavenged by reactive oxygen species (Radi, 2004). NO reacts with superoxide to form peroxynitrite (Radi and Alvarez, 2003). Peroxynitrite-derived secondary radicals, in turn, react with tyrosine residues in proteins to form nitrotyrosine (Pietraforte et al. 2003). As shown in Figure 7, there was a dramatic

increase in nitrotyrosine formation in the livers of rats fed the MHFD compared to rats on the control diets, which was evident as early as week one. These results suggest that a major portion of the decrease in hepatic NOx can be explained by a MHFD-induced increase in formation of reactive oxygen species (ROS). The MHFD-induced increase in ROS in the liver is consistent with other observations involving the effect of high fat diets of varying fat composition on various tissues (Galili et al. 2007; Roberts et al., 2003; Mantena et al. 2008).

In conclusion, the consumption of a MHFD caused circulating levels of nitric oxide to decline within two days of the onset of dietary treatment. Furthermore, the MHFD decreased levels of stable NO metabolites and eNOS expression in a tissue specific and time-dependent manner. The effect of the MHFD was particularly dramatic in the liver. The decrease in hepatic NOx appears to be due primarily to the scavenging effect of ROS and to a lesser extent to a decrease in eNOS activity.

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Figure 1

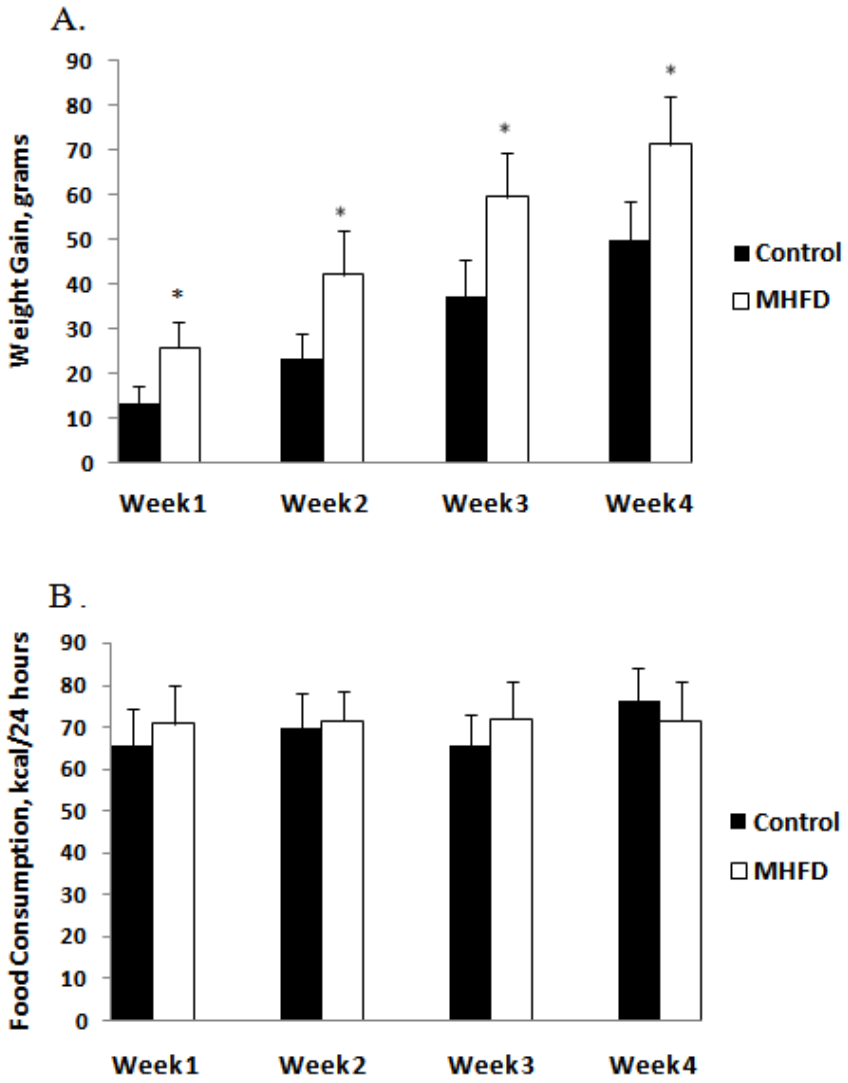

A.
Weight Gain, grams
90
80
70
60
50
40
30
20
10
0
Week1
Week2
Week3
Week4
Control
MHFD
B.
Food Consumption, kcal/24 hours
90
80
70
60
50
40
30
20
10
0
Week1
Week2
Week3
Week4
Control
MHFD

Figure 1. Weight gain and caloric intake by rats fed the control diet and moderately high fat diet (MHFD). A, Weight gain refers to the cumulative weight gained from the start of the experiment through the indicated week. B, Caloric intake was measured over a 24-hour period at weekly intervals. N = 8 rats per group. *P <0.05 compared to corresponding control group.

Figure 2

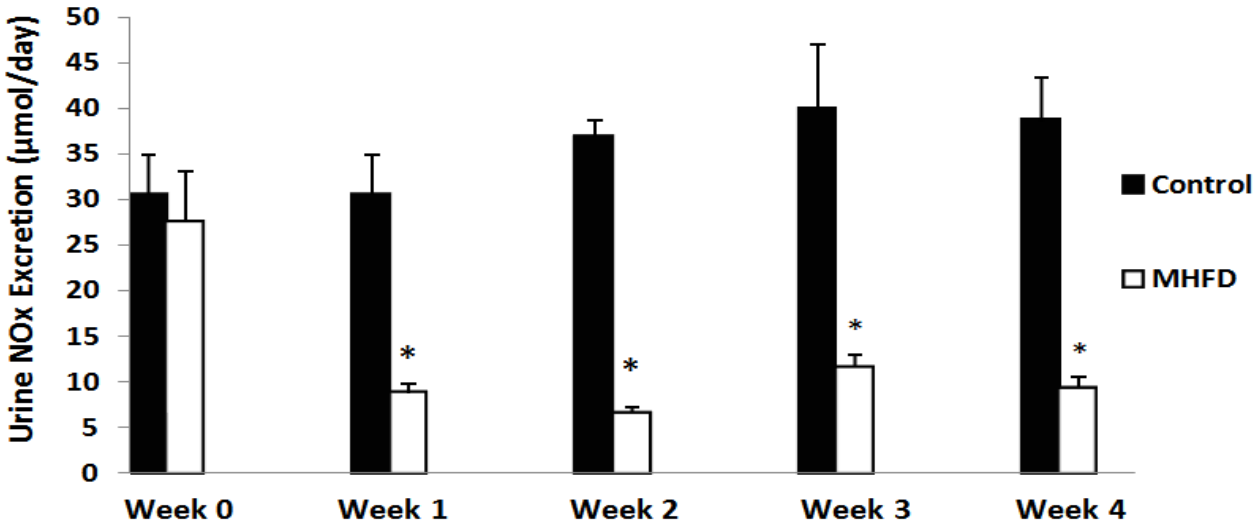


Figure 2. Urine excretion of stable NO metabolites (NOx) by rats fed the control diet and moderately high fat diet (MHFD). Twenty –four hour urine samples were collected from rats housed in metabolic cages at the end of the indicated week. At week 0 when baseline measurements were obtained, all rats were being fed the control diet. N = 8 rats per group. *$p < 0.05$ compared to rats on the control diet at the indicated week.

Figure 3

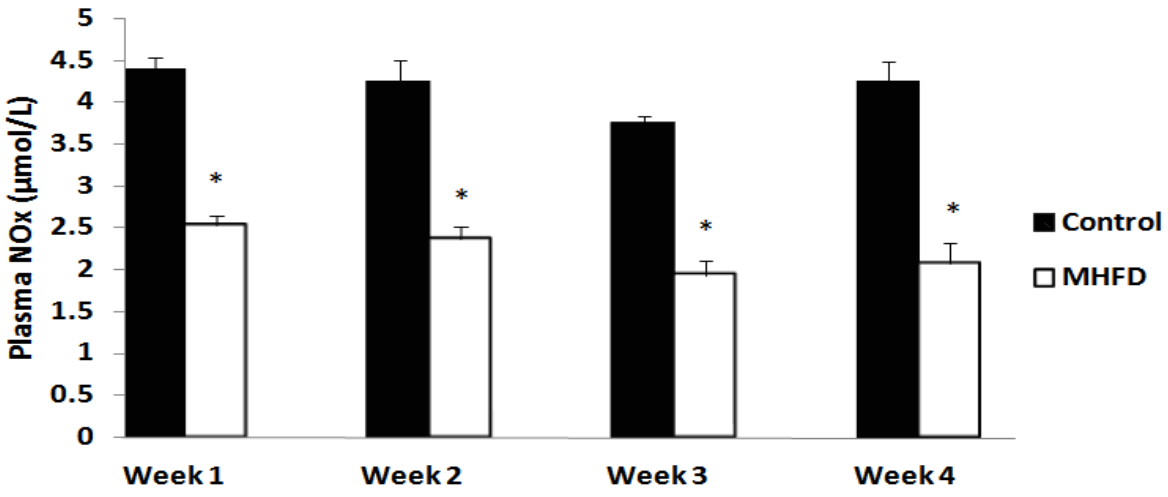
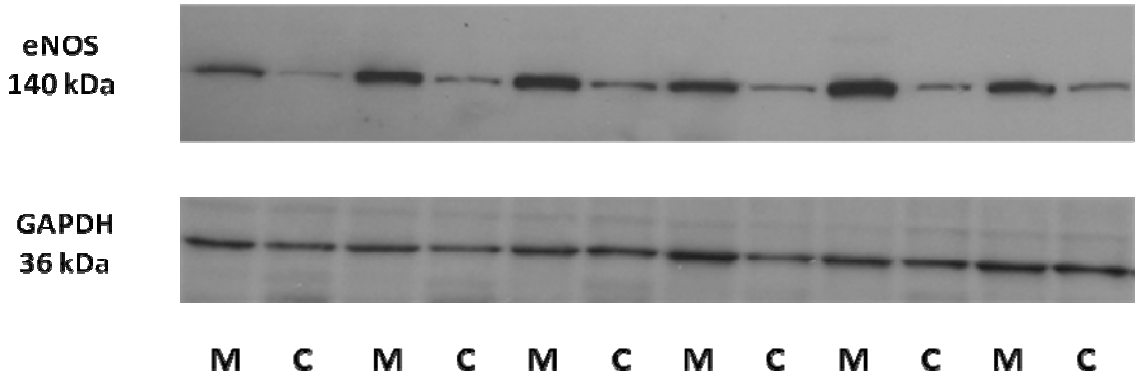


Figure 3. Plasma levels of stable NO metabolites (NOx) of rats fed the control diet and moderately high fat diet (MHFD). Plasma NOx levels were determined at weekly intervals beginning one week after the two groups of rats began consuming their assigned diets. N = 8 rats per group. *$p<0.05$ compared to rats on the control diet at the indicated week.

Figure 4A


eNOS
140 kDa
GAPDH
36 kDa
M C M C M C M C M C M C

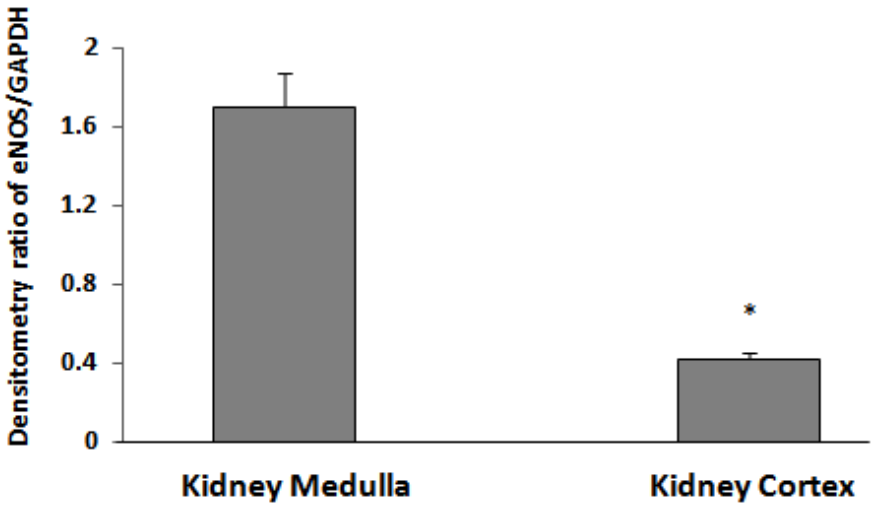

Densitometry ratio of eNOS/GAPDH
2
1.6
1.2
0.8
0.4
0
*
Kidney Medulla
Kidney Cortex

Figure 4B

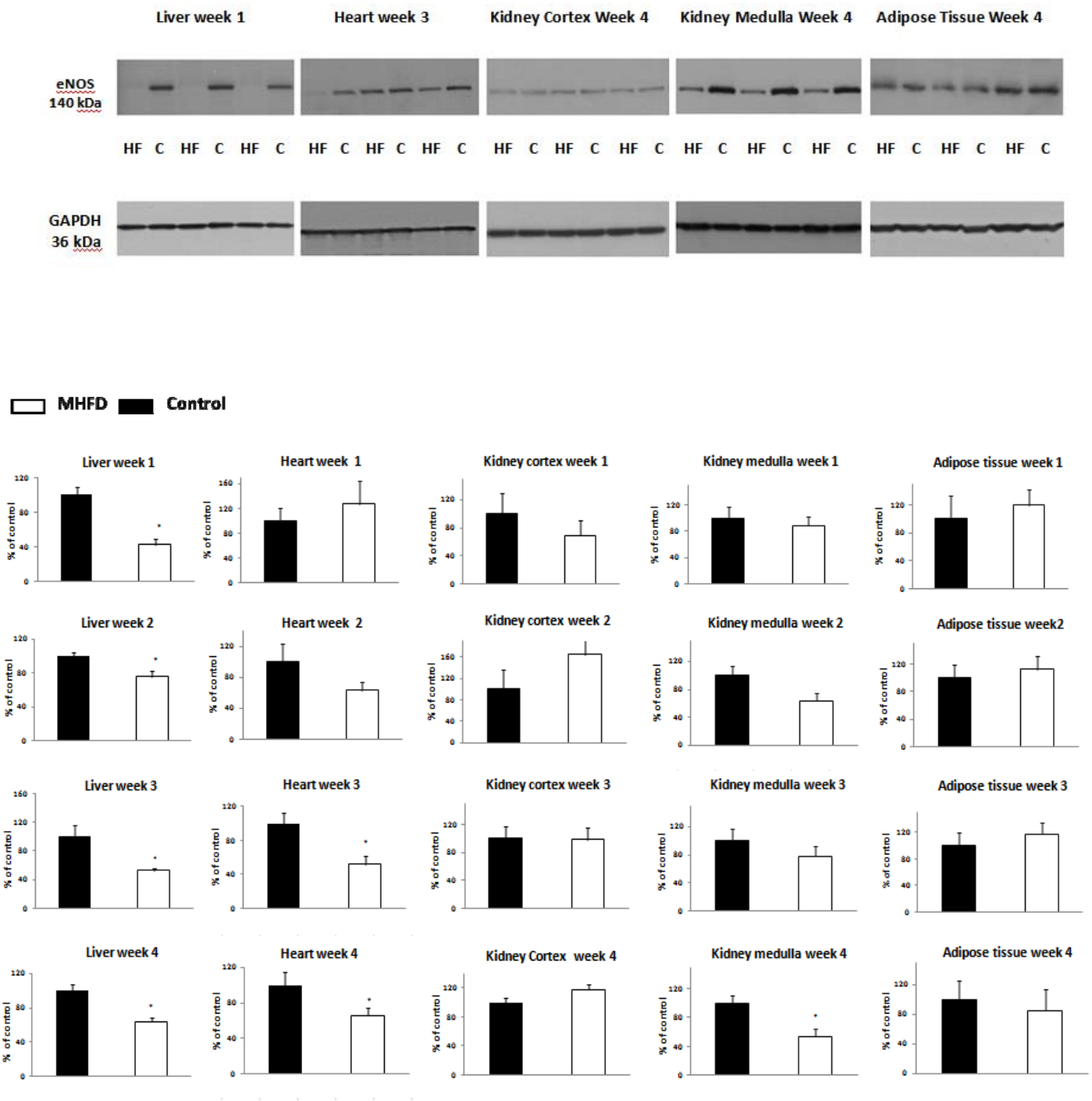

Liver week 1
Heart week 3
Kidney Cortex Week 4
Kidney Medulla Week 4
Adipose Tissue Week 4
eNOS
140 kDa
HF C HF C HF C HF C HF C HF C HF C HF C HF C HF C HF C HF C HF C HF C HF C
GAPDH
36 kDa
MHFD
Control
Liver week 1
Heart week 1
Kidney cortex week 1
Kidney medulla week 1
Adipose tissue week 1
Liver week 2
Heart week 2
Kidney cortex week 2
Kidney medulla week 2
Adipose tissue week2
Liver week 3
Heart week 3
Kidney cortex week 3
Kidney medulla week 3
Adipose tissue week 3
Liver week 4
Heart week 4
Kidney Cortex week 4
Kidney medulla week 4
Adipose tissue week 4
% of control

Figure 4. Endothelial nitric oxide synthase (eNOS) expression in tissues from rats fed the control diet and moderately high fat diet (MHFD). A, Western blot comparing eNOS expression in the medulla (M) and cortex (C) of kidneys from 6 rats that were fed the control diet. GAPDH was used for a loading control. In the associated graph, the relative intensity of the eNOS band is divided by the relative intensity of the corresponding GAPDH band. *$p<0.001$ compared to eNOS expression in the kidney medulla. B, Representative Western blots of tissues obtained from rats fed control (C) and moderately high fat (HF) diets are shown. Bar graphs represent the mean $\pm$ SE of eNOS expression for the indicated tissue obtained from 8 rats that were fed control diet (solid bar) or moderately high fat diet (open bar). eNOS expression was assessed for each tissue at weekly intervals over a period of 4 weeks. Data are presented as a percent of the control value for the specific tissue at the indicated time. *$p<0.05$ compared to tissues from rats on the control diet at the indicated week.

Figure 5

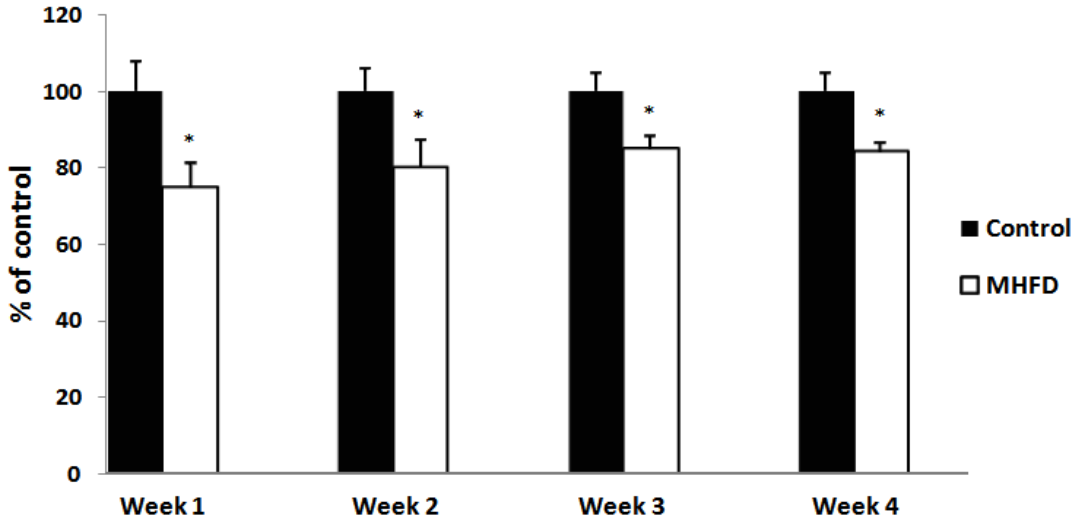


Figure 5. NOS activity in livers from rats fed the control diet and the moderately high fat diet (MHFD). NOS activity was measured at weekly intervals over a period of 4 weeks. N = 8 rats per group. *$p<0.05$ compared to control at the indicated week.

Figure 6

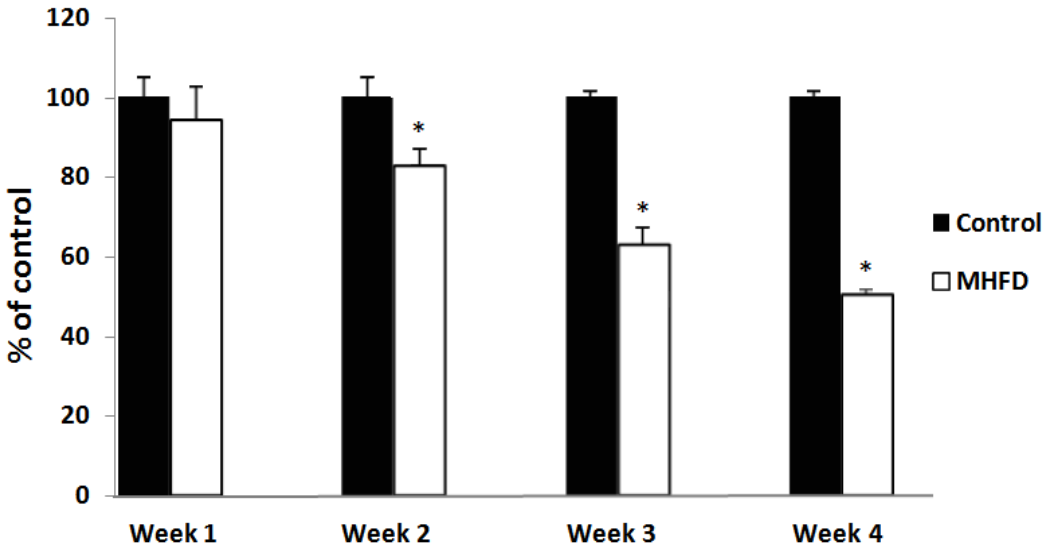


Figure 6. Liver content of NOx in rats fed the control diet and moderately high fat diet (MHFD). NOx content was measured at weekly intervals over a period of 4 weeks. N = 8 rats per group. *$p<0.05$ compared to control at the indicated week.

Figure 7

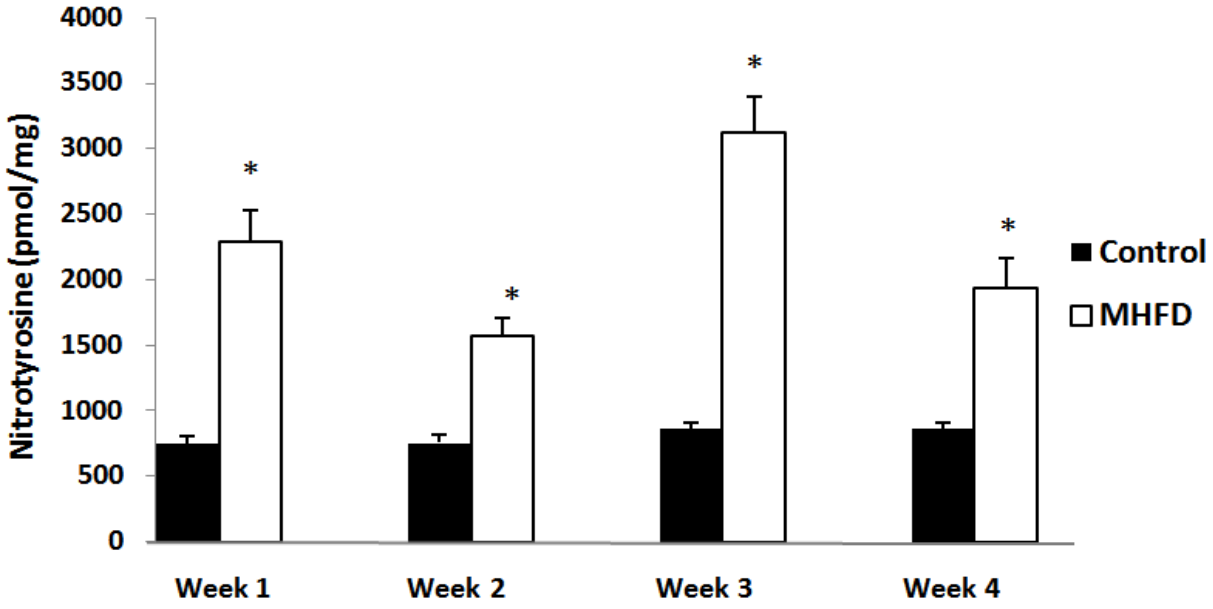


Figure 7. Nitrotyrosine formation by livers of rats fed the control diet and moderately high fat diet (MHFD). Liver nitrotyrosine content was measured at weekly intervals over a period of 4 weeks. N = 8 rats per group. *$p<0.05$ compared to control at the indicated week.

Figure 8

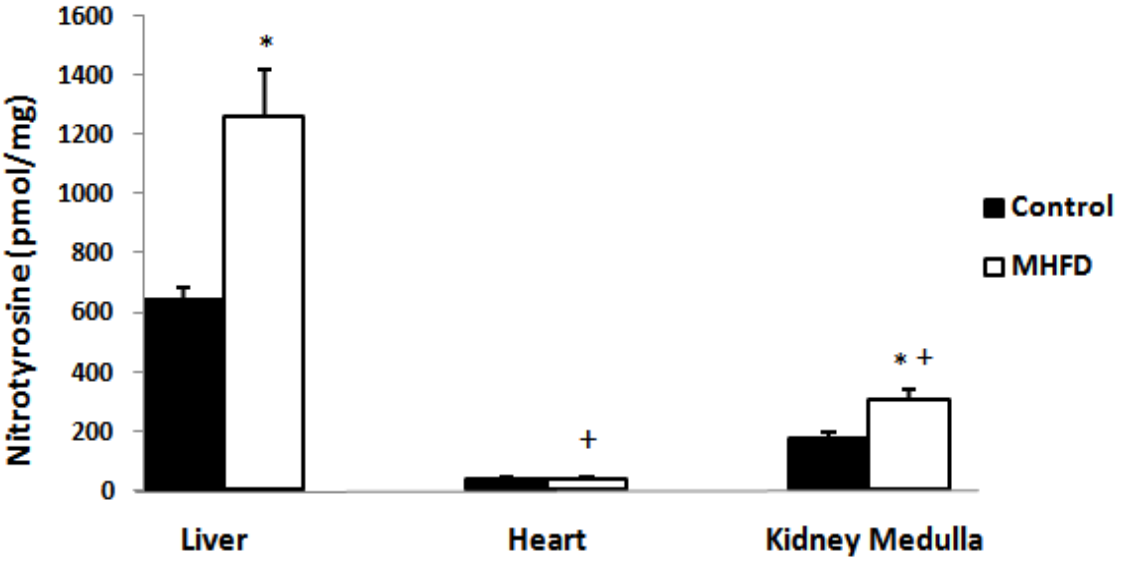


Figure 8. Comparison of nitrotyrosine formation by the liver, heart and the kidney medulla of rats fed the control diet and the moderately high fat diet (MHFD) for a period of 4 weeks. N = 7 rats per group. *$p<0.05$ compared to the control value for the indicated tissue; $^{+}p<0.05$ compared to nitrotyrosine formation by liver from rats on the MHFD.

Table-1

Diet	Control gm%	High Fat gm%
Protein	**23.4**	**18.5**
Carbohydrates	**48.1**	**56.7**
Fat	**4.5**	**15.6**
Total kcal/g	**4.07**	**4.41**

Table-1. Energy composition of the control and moderately high fat diet (MHFD).

Macronutrient components are given in gm%.

Chapter III

Changes in water intake associated with the consumption of a moderately high fat diet

Abstract

The objective of the experiments presented in this chapter was to determine the effects of the short-term consumption of a moderately high fat diet (MHFD) on water intake. This research is based on an earlier observation from this laboratory that rats fed a MHFD over a prolonged period of time decreased their water consumption. We investigated the time required for water consumption to decline and the compensatory mechanisms that followed the reduction in water intake. We also addressed the issue of the relative water content of the different diets used in this study. Rats ingesting a MHFD drank less water than rats on standard rat chow or a milk-protein based control diet. Differences in water intake were statistically significant by day two and persisted for the duration of the study. There were minor differences in the water content of the diets which could account for a small fraction of the decreased water intake by the MHFD group. Rats on the MHFD adjusted to the decrease in water intake by decreasing urine output and increasing urine osmolality. In light of the fact that all of the rats in this study had free access to water, these renal adjustments to the decrease in water intake support the hypothesis that the MHFD alters drinking behavior. To test the hypothesis that a NO-associated mechanism was involved in inducing a change in drinking behavior, the expression of eNOS and

nNOS in the supraoptic and paraventricular nuclei were measured. No change in the expression of eNOS and nNOS was observed at 2 days or 2 weeks after the rats began eating the MHFD, suggesting that an alteration in the expression of constitutively-expressed NOS in these nuclei may not be a factor in MHFD-induced changes in drinking behavior.

Introduction

Appropriate water consumption is critical for maintaining the normal physiological functions of our body. Sufficient water intake along with normal kidney function keep the plasma volume in a favorable range and remove the toxic by- products generated by metabolic processes of our body (Rhoades and Tanner 2003a). Water consumption is controlled by several mechanisms. A water deficit can raise plasma osmolality, cause dryness of mouth and throat and decrease blood volume if it persists long enough. The major stimulus of thirst sensation is an increase of plasma osmolality, which is detected by osmoreceptor cells. These osmoreceptor cells are distributed within the brain, especially in areas such as the organum vasculosum of the lamina terminalis and subfornical organ (Caston-Balderrama et al. 2001). Osmoreceptor cells sense changes in plasma osmolality and relay impulses to the cerebral cortex, which generates the conscious sensation of thirst and promotes water consumption (Andersson et al.1982). Another alternative but less powerful stimulus is the dryness of the mouth and throat, which is the consequence of reflexes that decrease salivary and buccal gland secretion. Dryness of the mouth and throat can directly stimulate the thirst center in the anterior hypothalamus and increase water intake (Rhoades and Tanner 2003b). If water-deprivation is uncompensated, the plasma volume will drop. The resultant hypovolemia stimulates baroreceptors in the carotid sinuses and aortic arch. The stimulated baroreceptors will induce the release of renin and formation of angiotensin II, which acts

on neurons near the third ventricle to stimulate thirst sensation (Rhoades and Tanner 2003b).

Nitric oxide (NO) was first discovered as a messenger molecule in the central nervous system in 1988 (Garthwaite et al. 1988). It distributes unevenly in the brain. Because it is mostly formed on demand, NO is considered an unconventional neurotransmitter (Bruhwyler et al. 1993). Previous research suggests that NO plays several roles in the brain such as providing protection against neuron injury following cerebral hypoxia or ischaemia (Beckman 1991), learning and memory processes (Chapman et al. 1992), and the regulation of hormone release from pituitary and hypothalamus (Duvilanski et al. 1995; Kaiser et al. 1996).

Supraoptic and paraventricular nuclei have been shown to play a very important role in central thirst regulation. First, anatomical evidence indicates that the supraoptic and paraventricular nuclei receive afferents from each of the sensory circumventricular organs in the central thirst regulation system (Caston-Balderrama et al. 2001). Second, NO has been shown to directly modulate supraoptic and paraventricular nuclei activity (Stern et al. 2003; Ventura et al. 2008). NOS in the supraoptic or paraventricular area has been linked to the regulation of drinking behavior. One recent study showed that water-deprivation induced nNOS expression in the paraventricular nuclei of rats (Ryu et al. 2008). In another experiment, the intracerebroventricular injection of NG-nitro-L-arginine methyl ester (L-NAME), an nonspecific NOS inhibitor, attenuated water consumption in water-deprived rats (Liu et al. 1998). The same group of researchers also showed that intracerebroventricular administration of L-NAME attenuated water

consumption induced by either osmotic or hypovolemic stimulation (Liu et al. 1996). These data suggest that NO may play an important role in regulating drinking behavior.

Experiments from several laboratories have shown that rats fed a moderately high fat diet exhibit a decrease in constitutive NOS expression (Roberts et al. 2003, 2005), NOS activity, and the presence of stable NO metabolites in the urine (Morrison et al. 2007). An earlier observation from our laboratory showed that rats fed a moderately high fat diet over a prolonged period of time decreased their water consumption. The objective of the present study was to determine the effects of short-term consumption of a moderately high fat diet on water intake. The following questions were addressed in this study. How quickly does the decrease in water intake occur? Is there a corresponding decrease in urine output? Do rats respond to decreased water intake by forming urine that is more concentrated? Is the decrease in water intake due to differences in the water content of the control and high fat diets? Does the high fat diet cause a decrease in NOS expression in the supraoptic and paraventricular nuclei?

Methods

Animals: Seven-week-old female lean Zucker rats were purchased from Charles River Laboratories (Wilmington, MA, USA). They were allowed to acclimate for one week in the Marshall University Animal Resourses Facility before the start of the experiment. They were housed, 2 rats per cage, in plastic cages with corn chip bedding in a room with a 12 hour light/dark cycle and an ambient temperature of 23±2˚C. All procedures using

animals were reviewed and approved by the Marshall University Institutional Animal Care and Use Committee.

Research design: In one experiment, 24 rats were randomly selected and divided into 2 groups. One group was fed a standard balanced rodent diet (Rodent Diet 5001, Lab Diet, Brentwood, MO) and another group a moderately high fat diet (formula D12266B, Research Diets, New Brunswick, NJ). Rats were put in stainless steel metabolic cages for a 24 hour adjustment period before any data were collected. After the rats were acclimated, 24-hour base-line data were collected. The rats were placed on control or high fat diets for the remainder of the study. Data for body weight change, urine volume, urine osmolality, and water and food consumption were collected. Six rats from each group were randomly selected and sacrificed at day 2. The remaining rats were sacrificed at the end of 2 weeks. All rats were anesthetized with an intraperitoneal injection of ketamine HCl: xylazine (45:5 mg/kg) and then euthanized by cardiac puncture. Rats were decapitated after cardiac puncture and brains were removed. Brains were sliced with a vibrating microtome (LE12 7LZ Campden Instruments LTD, Lafayette, IN) to a thickness of 400 to 500 μm. Brain tissues were harvested from supraoptic and paraventricular areas by micropuncture, and immediately placed in liquid nitrogen. They were then stored at -80˚C until processed for Western Blotting analysis.

In a second experiment, 16 rats were randomly selected and divided into 2 groups. One group was fed the standard rodent diet (Rodent Diet 5001, Lab Diet, Brentwood, MO), and the other group was fed the moderately high fat diet (formula D12266B, Research Diets, New Brunswick, NJ). All the rats were placed in metabolic cages to collect daily

samples for measuring urine output, urine osmolality, food intake and water consumption after the 24 hour acclimatation period. Base-line data was collected during a 24 hour interval before the start of the experiment. Twenty-four hour urine samples were collected at 4˚C. This experiment was continued for 7 days. All rats were sacrificed as described above at the end of the experiment.

In a third study, 24 rats were randomly selected and divided into 2 groups. One group containing 16 rats, was fed the moderately high fat diet (formula D12266B, Research Diets, New Brunswick, NJ) and the other group of 8 rats was fed a rodent diet as a standard control in which the protein came from casein (formula D12489B, Research Diets, New Brunswick, NJ). This is the same source of protein that is present in the MHFD. The research design and data collection were the same as described for study two.

Western Blotting: Tissue was homogenized in ice-cold RIPA buffer (Thermo Scientific Cat # 89901, Waltham, MA) with protease inhibitors (Complete Mini, Roche Diagnostics, Mannheim, Germany) by ultrasonic homogenizers (4710 Cole Parmer Instrument Co. Vernon Hills, IL). The homogenate was centrifuged at 12,000×g for 10 min at 4˚C to remove cellular debris. The supernatant was used for Western Blot analysis. Protein concentrations were determined using a Nanodrop Spectrophotometer (Thermo Scientific, Waltham, MA). The samples were separated on a SDS-polyacrylamide gel (10%) and transferred to a nitrocellulose membrane. The membrane was blocked in PBS containing 5% milk overnight at 4˚C. The membrane was probed with monoclonal anti-eNOS and anti-nNOS antibody (Transduction Laboratories Lexington, KY 1:2000) for 1

hour at room temperature. After 3 washings with PBS-Tween, the membrane was incubated with anti-mouse horseradish perioxidase-conjugated immunoglobuin antibody for 1 hour. eNOS and nNOS were detected by incubating the membranes with reagents from the ECL advance Western Blotting detection kit (Amersham life Science, Buckinghamshire, UK) and exposing them to films (Thermo Scientific, Rockford, IL).

Urine osmolality: Osmolality of urine samples was measured with an osmometer (Vapro pressure osmometer 5520, Wescor Inc, logan, UT) after the samples were diluted 1:10 to 1:20.

Water content of food: Food pellets were ground and passed through a sieve. 1 gram of grains from each type of pellet was weighed and placed in a small glass vial without a lid. Vials containing the three different diets were placed in glass jars containing desiccant. The jars were loosely sealed with lids and placed on a dry bath incubator which was maintained at 75˚C to 85˚C. The vials were weighted every 4 hours until their weights were stable for 8 hours.

Statistics: Data are expressed as means ± SEM. Western Blot data is expressed as a percent of the corresponding control. Statistical differences among means were determined by a T-test. Differences were determined to be statistically significant at $P \leq 0.05$. All the statistical analysis was performed using SigmaStat software (Jandel Scientific Software, SanRafael, CA).

Results

Water consumption: The data presented in Figure 1 were collected from rats maintained on the standard rat chow control diet (Diet 5001) or the moderately high fat diet (MHFD) for a period of one week. After the rats were allowed to adjust to the metabolic cages for a period of one day, a 24-hour baseline measurement of water intake was obtained (day 0). After this initial sample was collected, half the rats were placed on the MHFD and the other half remained on the control diet. By day two, rats on the MHFD were drinking significantly less water than rats on the control diet. This difference persisted for the remainder of the study. Because protein in standard rat chow comes from grain and protein in the MHFD comes from casein, an additional study was performed using the same protein source in both the control diet (D12589B) and MHFD (D12266B). In this study, all rats were fed the casein-based control diet for a week before the start of the experiment. They were also fed this diet during the period after they were placed in the metabolic cages and when the baseline measurement of water consumption was obtained for both groups. After baseline measurements were obtained (Day 0), one group of rats was placed on the MHFD. By day 2, there was a significant decline in water consumption by the group on the MHFD relative to the control group (Figure 2). This difference was still present two weeks later when an additional measurement was obtained.

Water content of the food: Since the higher water content of the MHFD could contribute to the decrease in water intake, the water content of each of the diets was determined. Results of this analysis indicate that the water content of the MHFD was slightly higher than that of the two control diets (Table 1).

Urine Volume: Figure 3 compares urine output by rats on standard rat chow (Diet 5001) and the MHFD over a period of 7 days. There was no difference in baseline data before the MHFD group was placed on the MHFD. Urine volume of rats fed MHFD dropped at day 2 compared to rats fed the control rodent diet 5001. This decline of urine volume persisted for the remainder of the experiment. This decline in urine output by rats on the MHFD paralleled the decrease in water consumption by this group (Figure 1).

By contrast, when compared to the casein-based control diet, there was no difference in urine output between the two groups when measured at one day, two days or two weeks after the rats started consuming the MHFD (Figure 4).

Urine osmolality: Urine osmolality was measured after samples were diluted 1:10 to 1:20 with water. Baseline urine osmolality of the rats in the control rodent diet 5001 and MHFD groups were the same before the start of dietary treatment. By day 4, there was a statistically significant difference in urine osmolality between these two groups of rats. This difference in urine osmolality persisted for the remainder of the experiment except on day 5 (Figure 5). When osmolality of urine from the MHFD rats was compared to that of rats on the casein-based control diet in a separated study, a statistically significant difference between the two groups was evident at day 2 (Figure 6). This difference was still evident after 2 weeks of dietary treatment.

eNOS and nNOS expression: The concentration of NO in supraoptic and paraventricular areas of the brain is thought to play a role in regulating drinking behavior. As shown in Figure 7, there were no differences in eNOS or nNOS expression induced by dietary

treatment in pooled tissue samples from the supraoptic and paraventricular nuclei after 2 days or 2 weeks of treatment.

Discussion

The consumption of a MHFD induced a decrease in water consumption that became apparent 2 days after the start of dietary treatment regardless of differences between the 2 control diets used in this study. This decline in water consumption persisted thereafter for the duration of the experiments. The 5001 control diet is standard Purina laboratory rat chow. This diet has a higher percentage of protein than D12489B. The protein in 5001 chow is derived from grains whereas the protein in the D12489B diet is milk protein (casein). The D12489B diet has the same protein source than the MHFD, which is the commercial version of the condensed milk diet. Both D12489B and the MHFD have similar protein contents, 16.4% and 18.5%, respectively.

The expected response to a decrease in water intake would be a reduction in urine output and the formation of more concentrated urine (Rhoades and Tanner 2003a). Urine output by rats eating the MHFD was less than urine output by rats consuming the 5001 control diet. This difference was statistically significant by day 2 and was evident throughout the study. Part of the difference of water intake could be due to the water content of the diet. The 5001 diet has about 1.5% water, whereas the MHFD has a water content of 4.8%. Based on food consumption data, rats on the 5001 diet ingested about 0.7 ml less water per day than the rats on the MHFD. Hence, the difference of water content in different

diet can only account for a small fraction of the decrease in water consumption by the rats on the MHFD.

When water consumption by rats on the MHFD is compared to water consumption by rats on the D12489B diet, differences are much less than when comparisons are made with rats on the 5001 control diet. Although, the two control diets were not compared in the same experiment, it is worth noting that baseline data for water consumption by rats on the D12498B experiment was about 20% lower than what is typically observed for rats on the 5001 control diet. In that the 5001 diet contains grain whereas the protein in D12489B comes from milk, it is likely that the 5001 diet contains a higher percentage of material that is not digestible. Consequently more water may be lost in the feces which will require a compensatory increase in the level of water intake. Because the D12489B diet is the commercially available control diet for the MHFD, the modest changes in drinking behavior seen when the D12489B diet was used as the control diet is a better reflection of changes induced by dietary composition.

When the D12489B diet was the control diet, a decrease in urine output did not accompany the decrease in water intake. There was, however, a statistically significant increase in urine osmolality in the MHFD group associated with the decreased ingestion of water. The osmolality of urine from rats on the MHFD was also greater than the osmolality of urine from rats on the 5001 control diet.

The intracerebroventricular administration of a NOS inhibitor, L-NAME, reduces the drinking response to osmotic stimulation and hemorrhage (Liu et al. 1996). We therefore

hypothesized that the MHFD would affect hypothalamic nuclei that control drinking behavior via a NOS-dependent mechanism. Both the supraoptic and paraventricular nuclei of the hypothalamus have been reported to express NOS (Ryu et al. 2008; Ueta et al.2002) and have been implicated in the regulation of thirst behavior. In the present study, there was no difference in NOS expression in pooled supraoptic and paraventricular samples. The data from these studies, however, may not be definitive. Because of limited tissue availability, we only measured the content of constitutively expressed NOS and did not measure NOS activity. Moreover, not enough tissue was harvested to measure iNOS expression or its activity.

Based on the results of these studies, we conclude that the consumption of a MHFD causes a decrease in water intake which, in turn, activates appropriate renal compensatory responses. Since all the rats had free access to water, these renal adjustments in response to decreased water intake support the hypothesis that the MHFD alters drinking behavior. Furthermore, experimental results suggest that the D12489B diet is a more appropriate control to use when assessing the effects of the commercial variant of the condensed milk diet on water balance.

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Figure 1

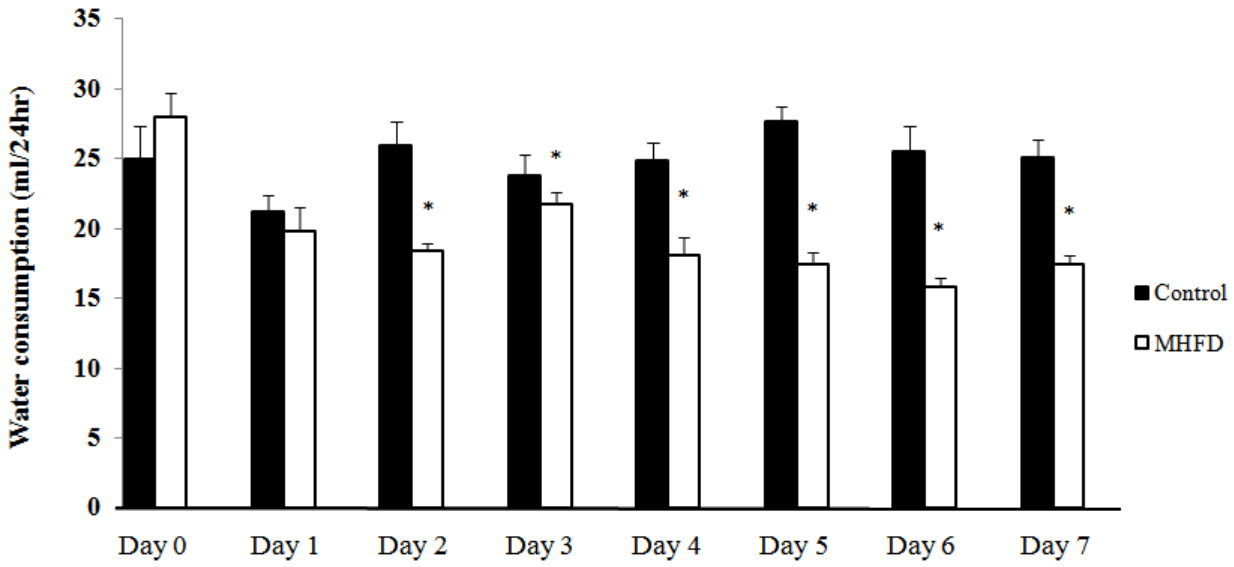


Figure 1. Water consumption (ml/24 hr) by rats eating a control rodent diet 5001 (solid bars) or a moderately high fat diet (open bars) for 7 days. Day 0 indicates the baseline data before the start of dietary treatment. Each bar represents the mean ±SEM (n=7) *$p<0.05$ compared to water consumption by rats on the control diet.

Figure 2

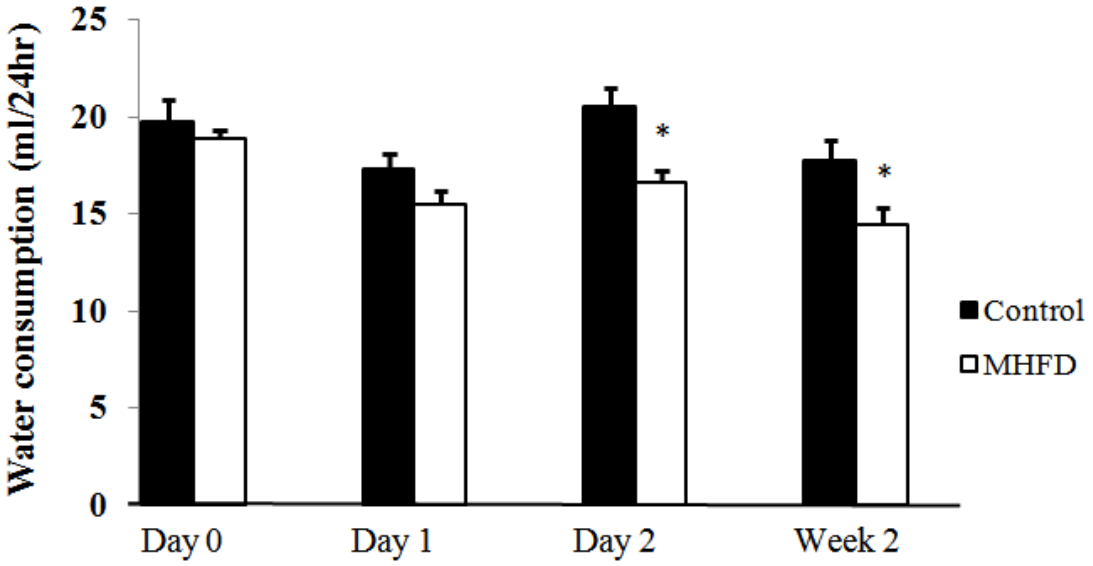


Figure 2. Water consumption by rats eating a control diet D12489B (solid bars) or a moderately high fat diet (open bars) for two days and two weeks. Both groups of rats were maintained on the control diet for one week before the start of the experiment. A baseline measurement of water consumption was obtained 24 hours before one of the groups was placed on the MHFD. Each bar represents the mean ±SE of water consumption by 8 rats. *$p<0.05$ compared to water consumption by rats on the control diet.

Figure 3

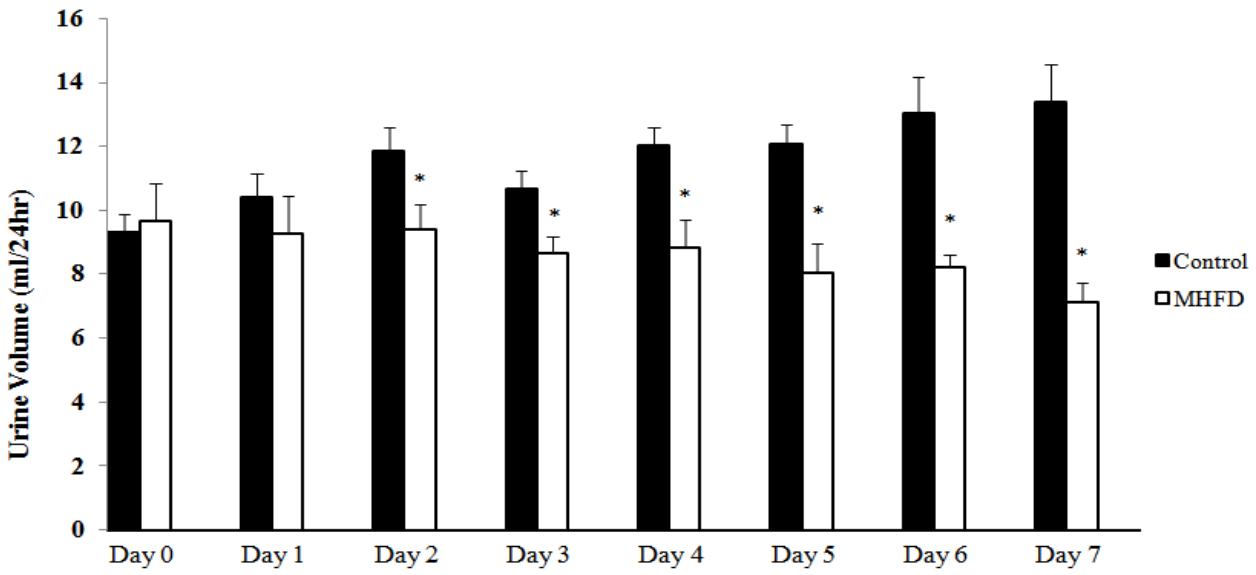


Figure 3. Urine output (ml/24 hr) by rats eating a control rodent diet 5001(solid bars) or a moderately high fat diet (open bars) for 7 days. Day 0 indicates the baseline data obtained during the 24 hour period before one group was placed on the MHFD. Each bar represents the mean ±SE of urine output for 7 rats. *$p<0.05$ compared to urine output by rats on the control diet.

Figure 4

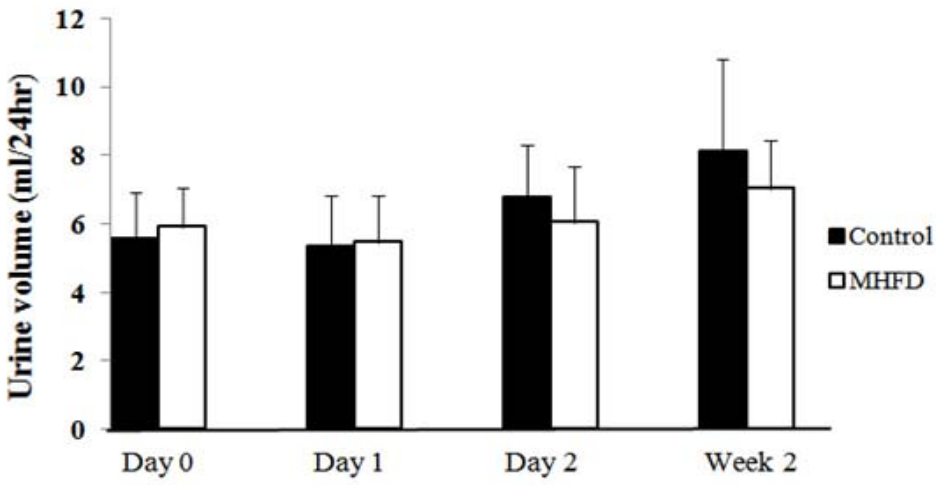


Figure 4. Urine output (ml/24 hr) by rats eating a control diet D12489B (solid bars) or a moderately high fat diet (open bars) for 2 days and 2 weeks. Day 0 indicates baseline data. Both groups of rats were maintained of the control diet for one week before the start of the experiment. A baseline measurement of urine output was obtained 24 hours before one of the groups was placed on the MHFD. Each bar represents the mean ±SE of urine output by 8 rats. *$p<0.05$ compared to urine output by rats on the control diet.

Figure 5

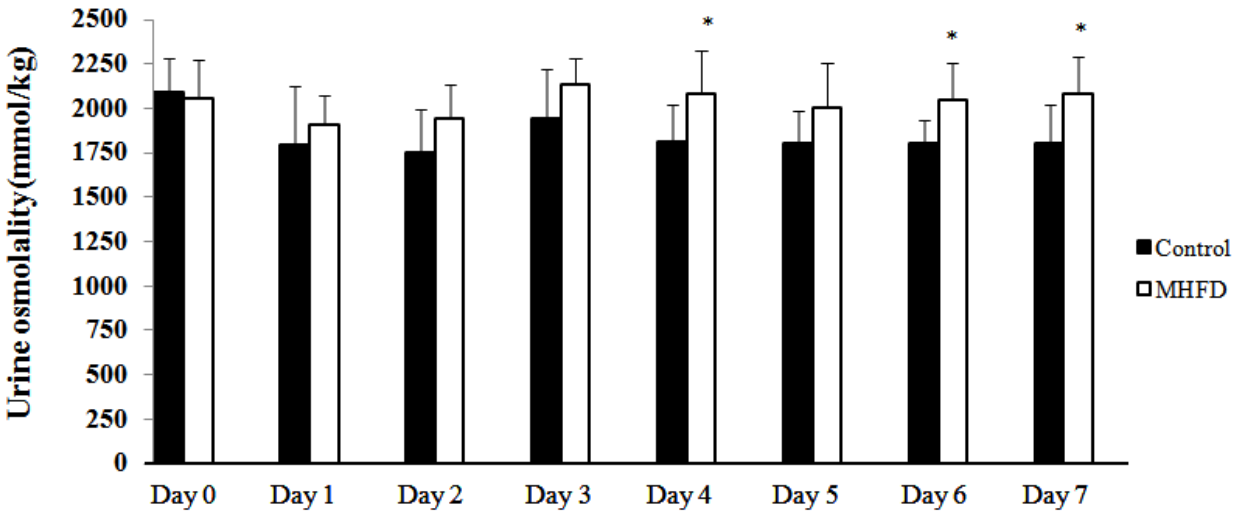


Figure 5. Osmolality of urine from rats eating a control rodent diet 5001(solid bars) or a moderately high fat diet (open bars) for 7 days. Day 0 indicates the baseline osmolality before the start of dietary treatment. Each bar represents the mean ±SE of the osmolality of urine from 7 rats. *$p<0.05$ compared to the osmolality from the urine from rats on the control diet.

Figure 6

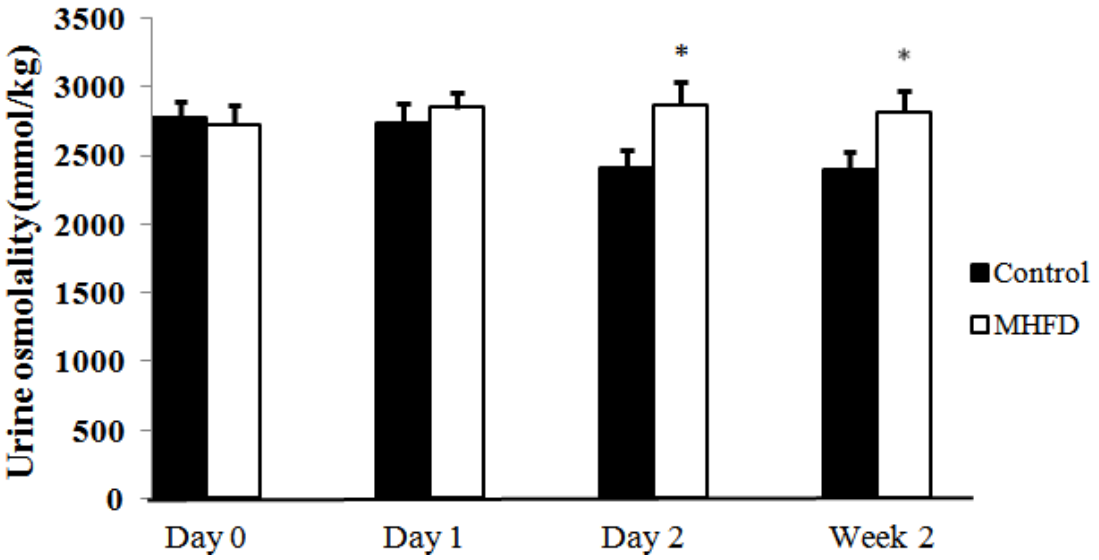
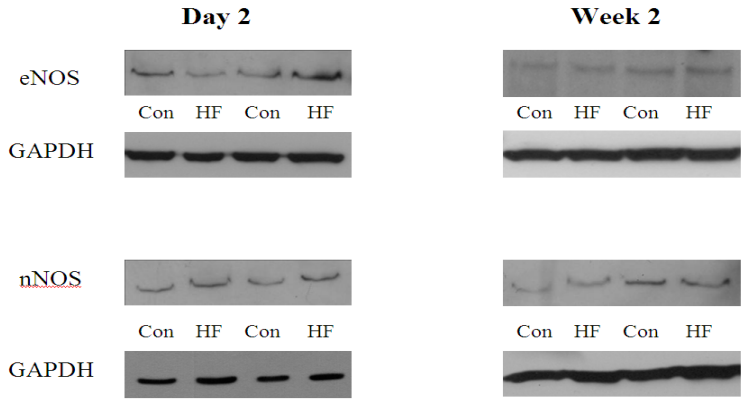
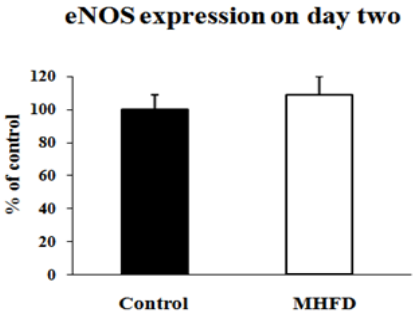
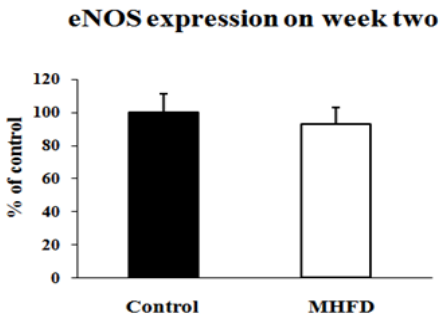


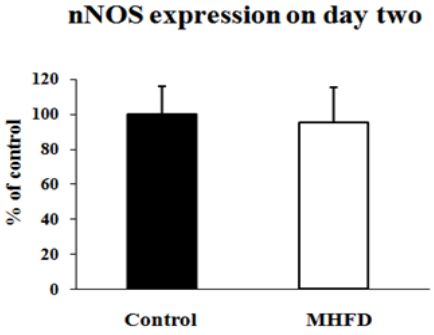
Figure 6. Osmolality of urine from rats eating a control diet D12489 (solid bar) or a moderately high fat diet (open bars) for 7 days. Baseline measurements were obtained as described in figures 2 and 4. Each bar represents the mean ±SE of urine osmolality for 8 rats. *$p<0.05$ compared to urine osmolality of rats fed the control diet.

Figure 7


Day 2
Week 2
eNOS
Con HF Con HF
GAPDH
nNOS
Con HF Con HF
GAPDH


eNOS expression on day two
% of control
120
100
80
60
40
20
0
Control
MHFD


eNOS expression on week two
% of control
120
100
80
60
40
20
0
Control
MHFD


nNOS expression on day two
% of control
120
100
80
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Control
MHFD

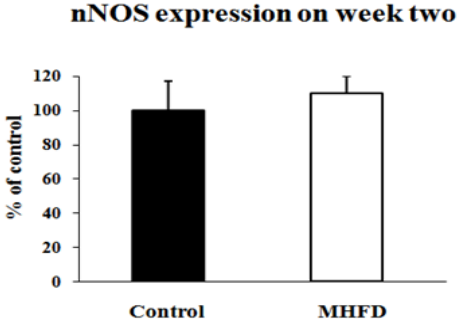
nNOS expression on week two
% of control
120
100
80
60
40
20
0
Control
MHFD

Figure 7. eNOS and nNOS expression in supraoptic and paraventricular areas of the brain. Representative Western Blots of hypothalamic nuclei from rats fed control rodent diet 5001(solid bars) and moderately high fat diet (open bars) are shown. Each bar represents the mean ±SE of eNOS/ nNOS expression in the indicated areas of brain, tissues were obtained from 6 rats in each treatment group. Western Blotting results are expressed as the densitometry ratio of eNOS/ nNOS to the loading control (GAPDH). Data are presented as a percent of the control value at the indicated time. *$p<0.05$ compared to tissues from rats on the control diet.

Table 1

Diet	Protein gm%	Carbohydrates gm%	Fat gm%	Calories kcal/gm	Water g%
Rodent diet 5001	23.4	48.1	4.5	4.1	1.5
D12489B	16.4	70.8	4.6	3.9	3.2
D12266B	18.5	56.7	15.6	4.4	4.8

Table-1. Energy composition and water content of three diets used in our studies.

Macronutrient components are given in gm%.

Chapter IV

Summary and Conclusion

General Discussion

The excessive consumption of dietary fat has become an important area of nutritional concern due to its negative impact on health. Multiple studies have demonstrated that the long-term consumption of diets high in fat content disrupts signaling pathways (Özcan et al. 2004), causes obesity (Dobrian et al. 2000), and contributes to the development of a number of obesity-associated diseases (Bian et al. 2008; Creager et al. 2003). Of particular concern is the effect of dietary fat on nitric oxide metabolism. Research has shown that the consumption of a high fat diet for several months decreases nitric oxide (NO) bioavailability (Roberts et al. 2005), which may contribute significantly to the pathogenesis of several diseases associated with altered NO bioavailability (Roberts et al. 2000; Baylis 2008; Bian et al. 2008). For example, long-term high fat consumption decreases circulating levels of stable NO metabolites and their excretion in urine (Roberts et al. 2000). Furthermore, NOS expression in several key organs is altered by the long-term ingestion of excessive dietary fat (Roberts et al. 2005). Other studies have shown that oral supplementation of the diet with arginine, a NO precursor, can attenuate pathological changes associated with the dietary fat overload (Hayashi et al. 2005). Moreover, reactive oxygen species which act as NO scavengers increase after the long-term consumption of a high fat diet (Dobrian et al. 2003).

In the experiments presented in this dissertation, the impact of consuming a moderately high fat diet (MHFD) during a 4 week period on NO bioavailability and NO production was investigated. The fat content of the MHFD used in this study was very similar to that of the typical U.S. diet. Twenty-four hour water consumption, urine output, food intake and urine content of stable NO metabolites were determined weekly. In addition, NOS expression in multiple organs was measured. Rats fed the MHFD significantly decreased their excretion of stable NO metabolites relative to rats on the control diet. Moreover, their plasma levels of stable NO metabolites also declined within a week of the onset of dietary treatment. eNOS expression was suppressed within one week in the liver, three weeks in the heart and four weeks in the kidney medulla, but not in adipose tissue or the kidney cortex, suggesting that the dietary fat induced decrease in NOS expression is time-dependent and organ specific. Among the organs that were investigated, the liver proved to be most sensitive to the MHFD. eNOS expression and NOS activity in the liver were significantly decreased by week 1 and stable NO metabolites within the liver dropped significantly by week 2. Moreover, liver nitrotyrosine formation increased significantly beginning at week 1. These data suggest that the short-term consumption of a MHFD decreases NO bioavailability by suppressing eNOS expression, inhibiting NOS activity and increasing the formation of NO scavengers.

Consumption of a MHFD for as few as 2 days decreased water consumption compared to water intake by rats on two different control diets. There were small differences in the water content of these diets, which may contribute in a minor way to the difference in water consumption. There was a difference in water intake by rats on the control diets, which suggest that components in the diet other than fat contributed to differences in

water intake. In rats on the MHFD, urine output decreased and urine osmolality increased. These changes persisted for the duration of the study, suggesting that the consumption of a diet high in fat content decreases drinking behavior. Because the supraoptic and paraventricular nuclei play important roles in regulating drinking behavior, eNOS and nNOS expression in this part of the brain were measured. No alterations in eNOS and nNOS expression were detected 2 days or 2 weeks after the dietary treatment was initiated.

In conclusion, the short-term consumption of a MHFD significantly reduces circulating NO levels by affecting mechanisms that regulate NO bioavailability, including a decrease in NOS expression and activity and an increase in the formation of reactive oxygen species which function as NO scavengers. In addition, the ingestion of a MHFD may affect other biological functions such as drinking behavior.

Future Work

Future studies to investigate the reduction in NO bioavailability associated with the increased consumption of dietary fat can be divided into four areas. First, the specific components in dietary fat that decrease NO bioavailability need to be identified. For example, saturated fats and unsaturated fats may play decidedly different roles in regulating NO bioavailability. Moreover, specific fatty acids derived from the digestion of dietary fats may have varied functions. Use of specific fatty acids contained in the MHFD to stimulate cultured cells may be a particularly productive area of investigation.

Second, the investigation of other pathways involved in the removal and recycling of NO maybe necessary to fully explain the decrease of NO bioavailability. Reversible interactions of NO with sulfhydryl or cysteine residues of proteins could be involved in the decreased bioavailability of NO. Third, the mechanisms through which dietary fats suppress eNOS expression and inhibit NOS activity need to be further investigated. Details of the transcriptional regulation of the eNOS gene are not fully understood. Perhaps dietary fats induce posttranslational modification of NOS, which may inhibit its activity. For example, dietary fat may upregulate caveolin expression, which can inhibit NOS activity. Finally, the question of whether or not the short-term effects of the MHFD on NO bioavailability are reversible needs to be addressed.

To further investigate the effects of the MHFD on the central regulation of water intake, paraventricular and supraoptic nuclei samples could be pooled from several animals in order to have enough tissue to determine whether NOS activity is being affected by dietary fat. Additionally, other areas of the brain that participate in the regulation of drinking behavior could be investigated with respect to NO production. Gonzalez-Lima et al. (1993) have shown that multiple areas of the brain outside of the hypothalamus are activated when rats engage in drinking behavior. Alternatively, signaling pathways that do not involve NO need to be investigated. For example, the intracerebroventricular administration of angiotensin II (Fleegal and Sumner, 2003) has been shown to promote drinking behavior. Perhaps, the local suppression of the renin-angiotensin system may be involved in mediating the reduction in drinking behavior associated with the consumption of a MHFD.

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Appendix

Date: Tue, 14 Apr 2009 09:04:29 +0100
From: richard.g.knowles@gsk.com Block Address
To: "Kan Huang" <huang6@marshall.edu>

Subject: Re: Use figure from your paper, permission needed

No problem, go ahead.
Regards, Richard

Dr, Knowles,

I would like to use figure 1.Domain structure of human nNOS, eNOS and iNOS from your paper "Nitric oxide synthases: structure, function and inhibition" published in Biochemistry Journal for my Ph.D. thesis. This figure is very important to my thesis, I hope you will grant me the permission to use it.

Thank you very much

Sincerely,

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EDUCATION

2004 Aug- Present	**Marshall University, Huntington, WV, USA** Ph.D. Candidate, Biomedical Sciences
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RESEARCH PROJECTS

The impact of the short-term consumption of a moderately high fat diet on nitric oxide production and bioavailability.

Drinking behavior change induced by short-term consumption of a high fat diet.

PUBLICATIONS

Huang K ., Huang Y., Frankel J., Addis C., Jaswani L., Wehner P.S., Mangiarua E.I. and McCumbee W.D. The short-term consumption of a moderately high fat diet alters nitric oxide bioavailability in female lean Zuck rats. In preparation.

Huang Y., Huang K., Boskovic G., Dementieva Y., Denvir J., Primerano D.A. and Zhu G.Z. Proteomic and genomic analysis of PITX2 interacting and regulating networks. FEBS Lett. 2009 Feb 18;583 (4):638-42.

CONFERENCE PRESENTATIONS

Huang K., Janswani L., McCumbee W.D. et al. The short-term consumption of a moderately high fat diet alters nitric oxide bioavailability in female lean Zuck rats. University of Kentucky Linda and Jack Gill Heart Institute Cardiovascular Research Day. Lexington, KY, 2008 October.

Huang K., Frankel J., McCumbee W.D. et al. The short-term consumption of high fat diet affects endothelial nitric oxide synthase in lean Zucker rats. The Obesity Society Annual Conference, Phoenix, AZ, 2008 October.

Huang K., Frankel J., McCumbee W.D. et al. A short-term consumption of high fat diet affects endothelial nitric oxide synthase in lean Zucker rats. Marshall Joan C. Edwards School of Medicine 2008 research day, Huntington, WV, 2008 March.

PROFESSIONAL MEMEMBERSHIP

American Association of Advanced Science

RESEARCH SKILLS

Protein: SDS-PAGE electrophoresis, western blot, immunoprecipitation, enzymatic and colorimetric assays, protein purification, immunohistochemistry, ELISA.

DNA/RNA: RNA extraction and purification, RT-PCR.

Animal: Metabolic cage, blood pressure measurement, animal surgery and handling, tissue slides preparation.

REFERENCE

Reference available upon request.