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NIACIN AND MICROVASCULAR ENDOTHELIAL CELL RESPONSE TO FATTY ACID EXCESS AND HYPOXIA

(Thesis format: Monograph)

by

Dominic Pang

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Niacin can reduce vascular disease risk, but its mechanism of action is controversial, and may not be dependent on systemic lipid modifying effects. This thesis tested the hypothesis that niacin directly improves endothelial cell function under lipotoxic and low oxygen conditions, as seen in ischemic conditions during metabolic syndrome, and investigated the potential mechanism involved. Human microvascular endothelial cell (HMVEC) survival was reduced by exposure to excess fatty acids under both normoxic and low oxygen conditions. Angiogenic function, as determined by tube formation on Matrigel, was impaired during fatty acid overload under either normoxic or low oxygen conditions. These effects were prevented by pretreatment with niacin, at a pharmacologically relevant dose, and appeared to be mediated by the niacin receptor, GPR109A, which we have recently shown to be expressed on human endothelial cells. In a mouse model of diet-induced obesity and metabolic syndrome, treatment with niacin improved functional recovery from acute ischemic injury. In conclusion, niacin improved HMVEC angiogenic function under lipotoxic and hypoxic conditions, effects that may translate to improved recovery from peripheral ischemia *in vivo*.

Keywords

Endothelial cells, nicotinic acid, metabolic syndrome, obesity, lipotoxicity, hypoxia, angiogenesis, GPR109A, hind limb ischemia.

Co-Authorship Statement

Palmitate β-oxidation in Figure 3.1 was performed with the help of Cindy Sawyez.

Plasma and liver lipid and enzyme measurements in Table 3.1 were performed through the Metabolic Phenotyping Laboratory in Robarts Research Institute by Cindy Sawyez and Brian Sutherland, and the London Health Science Center Core Facility.

Immunostaining of TA muscles was performed by Zengxuan Nong.

Vessel density and smooth muscle α-actin analyses in Figure 3.9 B & C were performed with the help of Jelena Toma.

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Abbreviations and Symbols

ABCA1	ABC transporter A family member 1
AHA/NHLBI	American Heart Association/National Heart, Lung, and Blood Institute
ANGPT1	Angiopoietin 1
AnnV	Annexin V
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCA	Bicinchonic Acid
BMI	Body mass index
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CETP	Cholesteryl ester transfer protein
CHOP	C/EBP homologous protein
CPT1	Carnitine palmitoyl transferase I
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

HAEC	Human aortic endothelial cell
HDL	High density lipoprotein
HIF-1	Hypoxia-inducible factor 1
HMVEC	Human microvascular endothelial cell
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cell
IDF	International Diabetes Federation
i.p	Intra-peritoneal
LDL	Low density lipoprotein
MCP-1	Monocyte chemoattractant protein-1
NA	Niacin/Nicotinic Acid
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NEFA	Non-esterified fatty acids
NF-κB	Nuclear factor κB
Nnt	Nicotinamide nucleotide transhydrogenase
NO	Nitric oxide
OA	Oleate/Oleic acid
Opti-MEM	Opti-minimum essential medium
PA	Palmitate/Palmitic acid

PAD	Peripheral arterial disease
PBS	Phosphate buffered saline
PI	Propidium iodide
PKC	Protein kinase C
PP2A	Protein phosphatase 2A
PPARγ	Peroxisome proliferator-activated receptor γ
PVD	Peripheral vascular disease
ROS	Reactive oxygen species
siRNA	Small interfering ribonucleic acid
TNFα	Tumour necrosis factor α
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low density lipoprotein
WD	Western diet

Chapter 1

1 Introduction

1.1 Obesity and Metabolic Syndrome, and their association with Ischemic Vascular Disease

Metabolic syndrome has become a highly prevalent complication of obesity globally, and its occurrence is continuing to increase. Since 1980, worldwide obesity has doubled. In 2008, more than 1.4 billion adults were overweight (World Health Organization, 2013). The increase in prevalence of metabolic syndrome corresponds most closely to the increase in consumption of lipogenic foods over this time frame. The composition and caloric content of commercially manufactured meals, and their relatively low cost have encouraged consumption of larger portions. (Sturm & An, 2014; Unger & Scherer, 2010).

Components of metabolic syndrome include elevated blood pressure, dyslipidemia, increased blood glucose, and central (visceral) obesity. Various diagnostic criteria for metabolic syndrome have been proposed by different organizations over the past decade. The International Diabetes Federation (IDF) and the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) have recently defined the criteria and cut-offs for metabolic syndrome. In order to be diagnosed with metabolic syndrome, one must have at least 3 of the 5 following criteria: elevated waist circumference, elevated triglycerides greater than 1.7 mmol/L, reduced high-density lipoprotein (HDL) less than 1.0 mmol/L, elevated blood pressure with systolic ≥130 and/or diastolic ≥85 mm Hg, or elevated fasting glucose of ≥100 mg/dL (Alberti et al., 2009). Whether central obesity (elevated waist circumference) is considered an absolute

requirement for diagnosis varies between proposed criteria. Obesity is defined as abnormal or excessive fat accumulation that may impair health, and is largely caused by an energy imbalance between calories consumed over calories used (World Health Organization 2013). In order to be considered obese, body mass index (BMI = weight [kg]/height^2 [m^2]) must be greater than 30 kg/m^2.

During obesity, excessive adipose tissue accumulation is directly related to increased adipocyte number and size. The role of adipose tissue in physiology and pathophysiology is complex since it is involved not only in lipid synthesis and storage, but also in secretion of different molecules. Adipocytes store excess lipids during increased fuel (fatty acids and glucose) availability in the form of triglycerides, which can be released by lipolysis in the fasted state to provide free fatty acids for energy to other organs (Suganami, et al 2012). Adipokines, such as leptin, are released in parallel to adipocyte expansion. Leptin acts on the hypothalamus to decrease food intake to limit over-nutrition, and can also minimize ectopic accumulation of lipids in cells other than adipocytes through leptin-induced fatty acid oxidation (Unger & Scherer, 2010). Adipocytes are also known to secrete proinflammatory molecules during obesity, such as tumour necrosis factor alpha (TNFα) and monocyte chemoattractant protein-1 (MCP-1) (Guilherme et al., 2008). This leads to macrophage infiltration into adipose (inflammation), and further contributes to TNF α and MCP-1 production and release (Campia et al., 2012). Both TNFα (Hotamisligil, 2003) and MCP-1 (Sartipy & Loskutoff, 2003) have been shown to induce insulin resistance in rodent models of obesity. Insulin resistance is increasingly recognized to play a pivotal role in the development of metabolic syndrome.

Adipose tissue inflammation can lead to uncontrolled lipolysis, through the actions of TNFα on adipocyte lipogenic and lipolytic pathways (Guilherme et al., 2008). The liver is sensitive to this increased flux of fatty acids from adipose, and responds with increased synthesis of very low density lipoproteins (VLDL) (Sniderman & Cianflone, 1995; Nikolopoulou & Kadoglou, 2012). Increased VLDL production also results from hepatic insulin resistance, as insulin is no longer able to suppress VLDL synthesis in this setting (Sparks et al., 2012). With chronic ingestion of lipogenic diets and the onset of obesity, increased circulating fatty acids and VLDL can cause lipids to be stored at sites other than adipose tissue, including skeletal muscle, heart, liver, and vasculature (Muoio & Newgard, 2006). The different organs affected by this ectopic lipid accumulation during obesity and metabolic syndrome can be seen in Figure 1.1.

Vascular diseases are the most common complications in individuals with obesity and metabolic syndrome (Campia et al., 2012; Van Gaal et al., 2006). Elevated plasma lipids can cause inflammatory responses in the endothelium and the subsequent recruitment of macrophages (Ross, 1999; Libby, 2012). The accumulation of both macrophages and lipids within arterial walls can cause atherosclerotic plaques in large to medium sized arties (Talayero & Sacks, 2011). The development of plaques can occlude arteries, and upon rupture can lead to coronary syndromes or myocardial infarctions (Ross, 1999). Extracoronary manifestations of plaques can also occur and cause peripheral artery disease (PAD), which is associated with decreased blood flow to the extremities and distal tissues. The restriction of blood flow to the periphery causes peripheral ischemia and in some severe cases, it can lead to critical limb ischemia requiring amputation (Ouriel, 2001; Teodorescu et al., 2013). Metabolic syndrome is associated

with higher incidence of ischemic stroke (Chen, et al, 2006), increased ischemic myocardial injury and impaired recovery of function after reperfusion (Liu & Lloyd, 2013), increased peripheral artery disease (Garg PK et al., 2014), and the worsening of peripheral ischemia (Gardner AW et al., 2006).

1.2 Endothelial Damage during Obesity and Metabolic Syndrome

Endothelial cells are a major regulator of vascular health and serve many physiological functions. The endothelium is a monolayer of cells covering the vascular lumen and plays an integral role in the regulation of vascular tone, platelet activity, leukocyte adhesion, thrombosis, vascular wall permeability, vasodilation and constriction, and vascular repair (Sena et al., 2013). Endothelial cells are metabolically active and can secrete vasoactive regulators such as nitric oxide (NO) (Grover-Páez & Zavalza-Gómez, 2009), which is the key endothelium-derived relaxing factor involved in the maintenance of vascular tone and reactivity. Endothelial cells are also critical for vascular repair and regeneration (Imrie et al., 2010). Therefore, in addition to vasodilation, the maintenance of endothelial cell capacity for vascular repair and regeneration is recognized as vital to the control of vascular disease, the most common complication of obesity and metabolic syndrome.

Endothelial dysfunction was first reported to be associated with obesity in 1996 by Steinberg et al. It has since been observed that endothelial dysfunction is also common during metabolic syndrome and precedes the development of vascular disease, including peripheral ischemia (Campia et al., 2012; Kim et al., 2012; Vita and Hamburg, 2010). Clinically, it refers to a decrease in endothelium-dependent vasodilation, characterized by

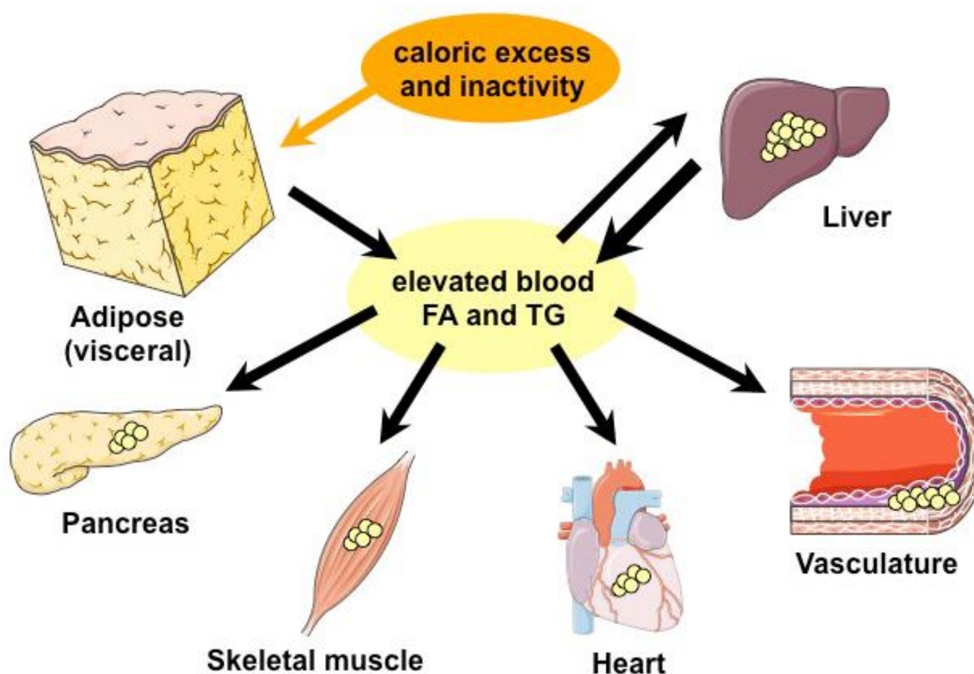


Figure 1.1. Organs affected during obesity and metabolic syndrome.

During caloric surplus coupled with a sedentary lifestyle, elevated circulating fatty acid levels eventually exceed the storage capacity of adipose tissue, resulting in the uncontrolled flux of non-esterified fatty acids (NEFAs) into the bloodstream and the development of a hyperlipidemic state. Hyperlipidemia causes hepatic insulin resistance, which increases hepatic production of VLDL into the circulation. Increased plasma concentrations of NEFAs and VLDL result in ectopic lipid accumulation in the pancreas, skeletal muscle, cardiac tissue, and the vasculature, causing tissue dysfunction. (Adapted from Muoio et al., 2006).

decreased NO bioavailability (Brevetti et al., 2008; Symons & Abel, 2013). Bigornia et al. (2010) showed that endothelial function was improved with weight loss in obese patients. Interestingly, their findings showed that metabolic changes were stronger determinants of vascular improvement than degree of weight reduction. This suggested that endothelial dysfunction was mediated by metabolic parameters during obesity, rather than by weight gain itself.

The response of endothelial cells to changes in their microenvironment is gradual and is dependent on the extent and intensity of the stressors. The endothelium initially responds to circulating stressors by modulating constitutive cellular functions, followed by endothelial dysfunction, and finally injury (Simionescu, 2007; Kim et al., 2012). Continued injury can ultimately lead to cell death, limiting the vascular repair and angiogenic functions of the endothelium (Imrie et al., 2010). When insults to endothelial cells, such as hyperlipidemia or hyperglycemia, are first presented the two constitutive functions of selective permeability and biosynthetic capacity are altered (Simionescu, 2007). Through a variety of *in vivo* experiments, it has been previously demonstrated that low density lipoproteins (LDL) or VLDL can accumulate in the subendothelium due to the altered permeability during obesity, as well as transcytosis of lipoprotein particles (Nielsen et al., 1992; Vasile et al., 1989). In conjunction with the change in permeability, endothelial cell conversion to a secretory phenotype has been observed, characterized by multiple well-developed biosynthetic organelles (Simionescu, 2007). In parallel to attaining a secretory phenotype, endothelial cells can also induce their expression of selective surface adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), in order to recruit monocytes and mount an inflammatory response (Grover-Páez &

Zavalza-Gómez, 2009). Endothelial cell dysfunction is present in the vasculature prior to the formation of an atherosclerotic plaque, serving as an earlier marker of vascular disease (Verma et al., 2003).

1.3 Lipotoxicity and Endothelial Cells

During obesity and metabolic syndrome, elevated circulating triglyceride-rich lipoproteins and free fatty acids can lead to lipid accumulation in non-adipose tissues (Figure 1.1). In many different cell types, this influences membrane structure, intracellular signaling, and energy homeostasis, and can ultimately lead to cell dysfunction and death. Collectively, this process is known as lipotoxicity (Brookheart et al., 2009). In all cell types, incorporation of fatty acids into triglycerides within cytosolic lipid droplets is the first line of defense in response to fatty acid overload (Garbarino & Sturley, 2009). Cells can also oxidize excess fatty acids, to varying degrees, through mitochondrial β-oxidation. It is only when these metabolic pathways are overwhelmed that lipotoxicity occurs. Endothelial cells may be particularly vulnerable to lipotoxicity because they are continually exposed to elevated concentrations of triglyceride-rich lipoproteins and free fatty acids during metabolic syndrome. Moreover, they are not metabolically programmed to process large amounts of lipids because they generate ATP primarily through aerobic glycolysis (Dagher et al., 2001; Helies-Toussaint et al., 2006). Pathways involved in lipotoxicity in endothelial cells are summarized in Figure 1.2, and are described in greater detail in the remainder of this section.

It has been generally observed that saturated fatty acids have adverse effects on cells. In contrast, unsaturated fatty acids are non-cytotoxic and may even protect against

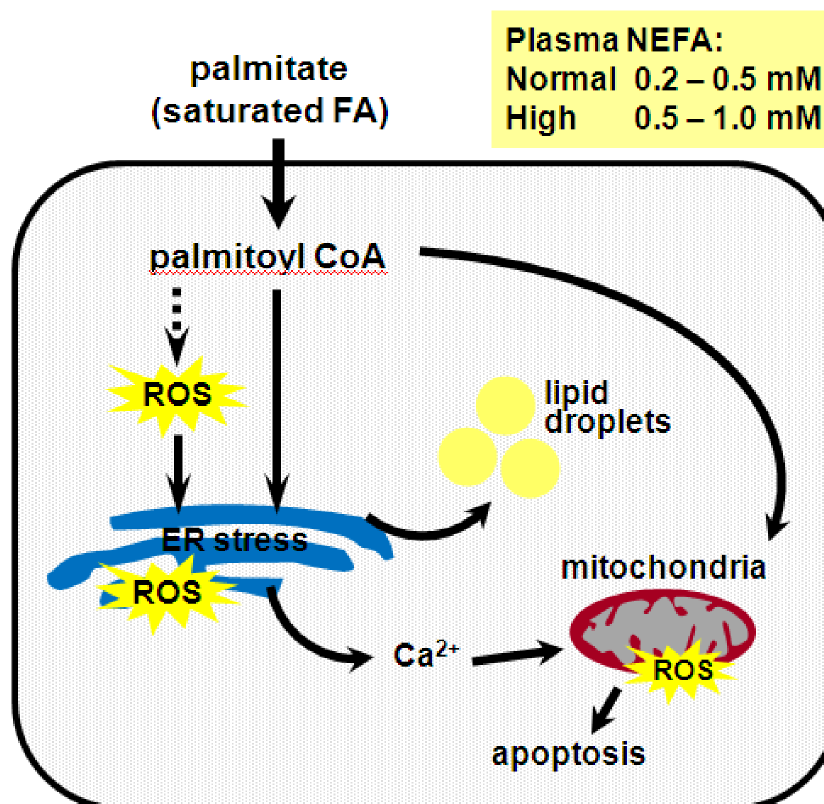


Figure 1.2. Roles of mitochondria and the ER in saturated fatty acid overload-induced cell death.

During exposure to excess saturated fatty acids, cellular capacities to store them as triglycerides (lipid droplets) and to catabolize them through β-oxidation (mitochondria) are overwhelmed. The resultant production of ROS, from several potential sources, can induce ER stress. Palmitate can also be rapidly incorporated into complex lipids in the ER membrane resulting in dramatic impairment of the structure and integrity of the ER. Both oxidative stress and altered ER composition and integrity result in the release of ER calcium stores, triggering cell death via mitochondria.

injury caused by saturated fatty acids. Previous studies in endothelial cells have demonstrated that saturated fatty acids can induce apoptosis in a dose dependent manner; whereas unsaturated fatty acids do not, and can even stimulate proliferation (Ciapaite et al., 2007; Staiger et al., 2006; Brookheart et al., 2009). Palmitate, the most common saturated fatty acid in western diets, has been shown to have lipotoxic effects in primary endothelial cell cultures and *in vivo* models (Yamagishi et al., 2002; Lu et al., 2013).

Oxidative stress due to reactive oxygen species (ROS) accumulation is commonly observed during palmitate overload in endothelial cells (Kim et al., 2012). Fatty acid induced oxidative stress can occur through protein kinase C (PKC)-dependent activation of NAD(P)H oxidase (Inogushi et al., 2000), and through the excess generation of ROS by-products of mitochondrial β-oxidation and ATP synthesis. Cellular levels of these ROS are normally controlled by enzymatic scavengers, however, limited or incomplete β-oxidation and altered [NAD^+]/[NADH] ratios likely contribute to excessive mitochondrial ROS production during fatty acid overload (Adam-Vizi & Chinopoulos, 2006). Endothelial cell dysfunction resulting from oxidative stress includes decreased NO bioavailability, through multiple mechanisms including generation of ONOO- anions (Campia et al., 2012). ROS can also activate nuclear factor κB (NF-κB) in endothelial cells causing its translocation to the nucleus, and subsequent activation of proinflammatory gene expression (de Winther etal., 2005). Thus excess saturated fatty acids can trigger dysfunction and death in endothelial cells through cellular generation and accumulation of reactive oxygen species (ROS).

During exposure to excess palmitate, there is an increase in toxic bioactive lipid metabolites, particularly ceramide (Symons & Abel, 2013). Ceramide is a lipid second

messenger involved in initiating apoptosis in response to stimuli (Brookheart et al., 2009). Palmitate is a preferred substrate for *de novo* ceramide synthesis, and increased *de novo* ceramide synthesis is a result of increased substrate availability (Listenberger et al., 2001). The studies of Zhang et al. (2012) revealed that *de novo* ceramide biosynthesis reduced protein phosphatase 2A (PP2A) association with the endothelial nitric oxide synthase (eNOS), which led to reduced agonist-stimulated eNOS phosphorylation and NO bioavailability in endothelial cells. Ceramide has also been demonstrated to increase NADPH oxidase activity and increase ROS production (Zhang et al., 2003). Thus palmitate can induce endothelial cell dysfunction and apoptotic cell death through the generation of ceramide.

Saturated fatty acid overload can also induce endoplasmic reticulum (ER) stress and cell death in several cell types (Brookheart et al., 2009). Excess palmitate is rapidly incorporated into ER membranes, leading to disruption of the ER membrane integrity, ER stress, and finally initiation of apoptosis (Borradaile et al., 2006). This phenomenon has recently been confirmed in endothelial cells by Lu et al. (2013) by analyzing the expression of ER stress markers, including C/EBP homologous protein (CHOP), in response to treatment with palmitate.

1.4 Hypoxia and Endothelial Cells

Extracoronary manifestations of atherosclerotic plaques can cause PAD, also known as peripheral vascular disease (PVD), which is associated with decreased blood flow to extremities and distal tissues. Restriction of blood flow to the periphery causes peripheral ischemia and in some severe cases, it can lead to critical limb ischemia

requiring amputation (Ouriel, 2001; Teodorescu et al., 2013). Loss of endothelial cell mediated vasodilation during endothelial dysfunction can also cause ischemia.

Under normal physiological conditions, endothelial cells mount an angiogenic response to hypoxic conditions induced by peripheral ischemia, in an attempt to restore blood flow (Manalo *et al*, 2005; Luo *et al,* 2012).

Furthermore, endothelial cells release NO upon exposure to acute hypoxia, which plays a major role in the local regulation of vascular smooth muscle tone. However, endothelial cell dysfunction decreases endothelium-dependent vasodilation, characterized by decreased NO bioavailability (Brevetti et al., 2008; Symons & Abel, 2013). Thus, endothelial cell dysfunction may prevent the release of NO for vasodilation, and from initiating angiogenesis during hypoxia.

An important endothelial response during hypoxia is the activation of transcription of many specific genes, regulated by hypoxia inducible factor 1 (HIF-1) (Parternotte et al., 2008). HIF-1 functions as a master regulator in response to cellular oxygen levels, undergoing conformational changes in response to oxygen concentrations (Ho et al., 2006). There are two subunits, HIF-1α and HIF-1β. The HIF-1β subunit is constitutively expressed and is oxygen independent. In contrast, HIF-1α is oxygen dependent and is rapidly degraded during normoxia by the ubiquitin-proteosome pathway (Ho et al., 2006). Exposure to low oxygen will inhibit the degradation of HIF-1α, allowing it to accumulate and translocate to the nucleus (Gao et al., 2012). Once in the nucleus, HIF-1α heterodimerises with HIF-1β to form an active transcription factor. HIF-1 plays a crucial role during angiogenesis in response to hypoxia by activating transcription of genes

encoding angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and angiopoietin 1 (ANGPT1) (Kelly et al., 2003).

In most cell types, mitochondria respond to altered oxygen levels through changes in electron transport chain complex activity (Bhatnager, 2003; Essop, 2007). Under low oxygen conditions, the electron transport respiratory chain runs less efficiently and cytochrome C has reduced capacity to trap oxygen. This phenomenon alters cellular redox potential and increases ROS production (Paternotte et al., 2008). Links between mitochondrial ROS generation and HIF-1 regulation during hypoxia have also been suggested (Cash et al., 2007; Pung et al., 2000). Hypoxia also diminishes cellular capacity for fatty acid β-oxidation, thereby limiting clearance of excess fatty acids through this metabolic pathway. Taken together, this suggests that combined hypoxia and fatty acid excess, as might occur during peripheral vascular disease associated with obesity and metabolic syndrome, would impair endothelial cell angiogenic function.

1.5 Regulation of Lipid Metabolism by Niacin

1.5.1 GPR109A-independent Effects

Niacin (nicotinic acid, vitamin B3) is one of the oldest lipid-lowering drugs and has been clinically used for decades since its discovery in 1955 (Altschul et al., 1995). Pharmacologic doses of niacin reduce all proatherogenic lipids and lipoprotein particles, including total cholesterol, triglycerides, VLDL, and LDL (Kamanna et al., 2013). Niacin also raises HDL cholesterol, thus improving overall lipid profiles in dyslipidemic patients, particularly those with the dyslipidemia characteristic of metabolic syndrome (elevated triglycerides and low HDL) (Creider et al., 2012; Goldberg et al., 2000). However,

clinical use of niacin is often avoided due to its cutaneous flushing effect which, although not harmful, limits patient compliance.

Several mechanisms have been proposed to explain the action of niacin to improve lipid profiles. The liver is one of the many target tissues for niacin. Using the human hepatocyte cell line (HepG2), Kamanna et al. (2013) recently demonstrated that niacin directly and noncompetitively inhibited microsomal diacylglycerol acyltransferase 2 (DGAT2), a key enzyme that catalyzes the final reaction in triglyceride synthesis. Inhibiting DGAT2 decreases triglyceride synthesis and thus its availability for VLDL assembly, resulting in decreased production of VLDL and its catabolic product, LDL (Creider et al., 2012). In support of this concept, Hu et al. (2012) showed in a small study in 39 dyslipidemia patients that extended- release niacin (2 g/day) treatment for 23 weeks significantly decreased liver fat content. Furthermore, they were also able to show that polymorphisms of DGAT2 were associated with a smaller reduction in liver fat content after niacin treatment.

As mentioned above, niacin also improves the lipid profile of dyslipidemic patients by increasing HDL. In the liver, niacin selectively inhibits the expression of hepatocyte β-chain ATP synthase, the hepatocyte HDL catabolism receptor, leading to reduced hepatic removal of HDL protein (Zhang et al., 2008). This results in increased apoAI HDL subfractions and helps drive reverse cholesterol transport (Villines et al., 2012). Niacin has also been shown to decrease cholesteryl ester transfer protein (CETP), which exchanges triglycerides in VLDL and LDL particles for cholesteryl esters in HDL particles (Van Der Hoorn et al., 2008). As plasma HDL levels are inversely associated with triglyceride levels, the reduction of triglyceride and CETP activity by niacin is

suggested as an indirect route to increase HDL cholesterol levels (Creider et al., 2012). Additionally, niacin may improve reverse cholesterol transport efficiency by increasing the expression of peroxisome proliferator-activated receptor γ (PPARγ) and ABC transporter A family member 1 (ABCA1) cholesterol transporters in macrophages and monocytes (Villines et al., 2012).

1.5.2 GPR109A-dependent Effects

Some of the effects of niacin are mediated through a cell surface receptor, GPR109A. This seven-transmembrane G-protein coupled receptor has a high affinity for niacin and was discovered by three separate groups in 2003 (Wise et al., 2003; Soga et al., 2003; Tunaru et al., 2003). GPR109A is endogenously activated by hydroxycarboxylic acids and couples to G_i-type G-proteins, resulting in inhibition of adenylyl cyclase (Ahmed et al., 2009). Since its discovery, it has been shown to be expressed by several tissues including adipose, intestinal epithelium, epidermal Langerhans cells, and several immune cell types (Ahmed et al., 2009). The adverse flushing side-effect of niacin has been reported to be a result of activation of GPR109A in Langerhans cells, leading to release of prostaglandins (Benyo et al., 2005). However, important to this thesis, GPR109A expression has not been previously reported in vascular endothelial cells.

Activation of GPR109A inhibits cyclic adenosine monophosphate (cAMP) accumulation in adipose tissue and mediates the anti-lipolytic effects of niacin *in vivo* (Tunaru et al., 2003). Decreased cAMP levels result in reduced hydrolysis of triglycerides to free fatty acids in adipose tissue (Offermanns, 2006), therefore limiting the substrate availability of fatty acids for hepatic triglyceride synthesis and subsequent assembly of VLDL and LDL. This phenomenon was further confirmed by Zhang et al. (2005). The

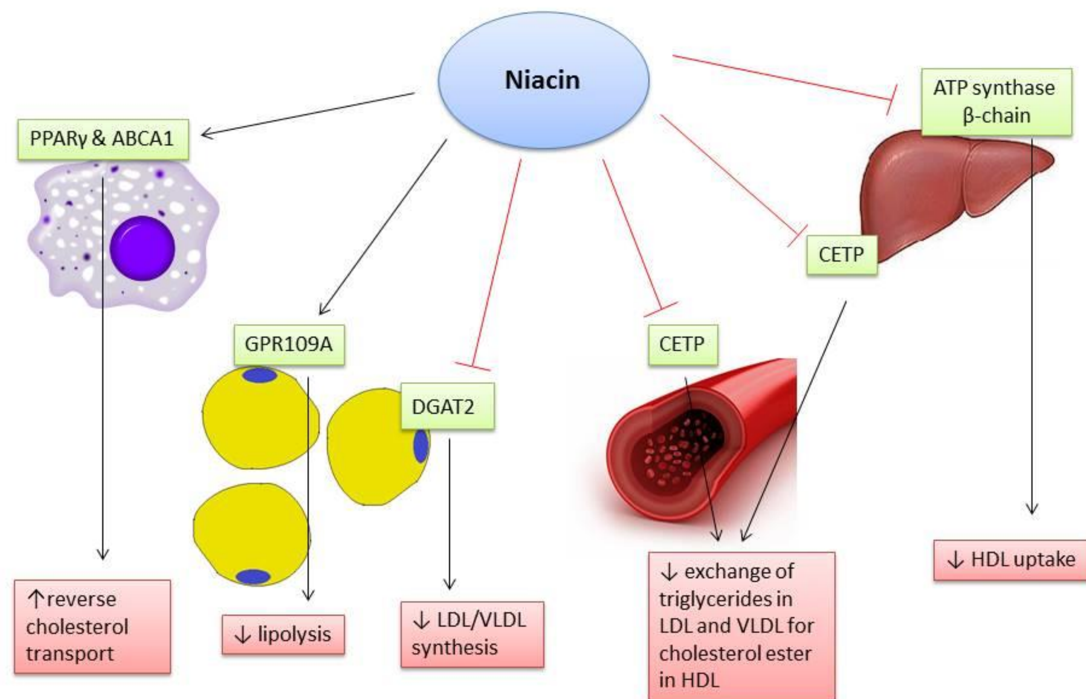


Figure 1.3. Mechanisms of action of niacin in regulating plasma lipids.

Niacin decreases LDL levels by: directly inhibiting DGAT2, a key enzyme for triglyceride synthesis; binding to receptor GPR109A to decrease lipolysis and free fatty acid flux to the liver. Niacin increases HDL levels by: selectively inhibiting hepatic ATP synthase β-chain, leading to reduced HDL removal; decreasing hepatic and plasma CETP, which exchanges triglycerides in VLDL and LDL particles for cholesteryl esters in HDL particles; increasing the expression of PPARγ and ABCA1 cholesterol transporters to improve reverse cholesterol transport. (Adapted from Creider et al., 2012)

combined inhibitory effects of niacin on DGAT2 in the liver and on lipolysis (via GPR109A) in adipose, results in decreased circulating VLDL and, subsequently, decreased exchange of triglycerides with HDL, leading to elevated levels of HDL cholesterol (Offermanns, 2006; Creider et al., 2012). However, a more direct role for the GPR109A-mediated effect of niacin in increasing HDL is supported by the fact that other, selective GPR109A agonists, such as acipimox also increase HDL cholesterol (Series et al., 1990).

Interestingly, the GPR109A-dependent effects of niacin in lipid lowering have recently been called into question. An extensive study with a GPR109A knockout mouse, and with agonists of GPR109A, showed that these mice retained the triglyceride and LDL lowering, and HDL raising effects of niacin (Lauring et al., 2012). In the same study, treatment of dyslipidemic patients with selective GPR109A agonists did not improve their serum lipid profiles, but did result in an acute lowering of plasma free fatty acids. A summary of the effects of niacin in regulating plasma lipids is depicted in Figure 1.3.

1.6 Regulation of Vascular Cell Function by Niacin

1.6.1 Immune Cells

Negative outcomes of recent clinical trials (Investigators A-H, 2011; Group HC, 2013) have undermined the clinical utility of niacin in combination therapies with statins in the long-term treatment of dyslipidemia in patients with characteristics of metabolic syndrome. Although treatment with niacin, like statins, is associated with worsening of blood glucose control in at risk patients (Ong et al., 2014), this adverse effect may be independent of activation of the niacin receptor, GPR109A. In fact, selective activation of

GPR109A in patients with type 2 diabetes mellitus has recently been shown to reduce serum glucose and noon-esterified fatty acids, and to improve insulin sensitivity, without the adverse skin flushing and gastrointestinal disturbances associated with niacin therapy (Dobbins et al., 2013). However, despite the disappointing results, niacin monotherapy improves vascular health in several patient populations (Creider et al., 2012), including those with the dyslipidemia commonly associated with metabolic syndrome (high triglycerides and low HDL) (Thoenes et al., 2007). Several studies have also suggested that the vascular benefits of niacin may not be dependent on its systemic lipid lowering effects (Chai et al., 2013; Digby et al., 2012; Lukasova et al., 2011; Wu et al., 2010).

Niacin can act on immune cells such as monocytes and macrophages, which express GPR109A (Ahmed et al., 2009). Recent evidence suggests that monocytes treated with niacin have reduced adhesion to endothelial cells (Tavintharan et al., 2011), and that neutrophil infiltration into vessel walls is decreased with niacin (Wu et al., 2010). Moreover, activation of GPR109A has been shown to inhibit MCP-1-induced recruitment of macrophages to atherosclerotic plaques. Niacin has also been shown to reduce the secretion of pro-inflammatory cytokines from macrophages (Lipszyc et al., 2013), and knockdown of GPR109A in monocytes results in a loss of the anti-inflammatory effect of niacin through the NF-κB pathway (Digby et al., 2012), all of which may further contribute to its anti-atherosclerotic effects. Interestingly, although niacin can act on macrophages via GPR109A, it loses its effect on foam cells, as a result of a downregulation of GPR109A as macrophages become lipid laden (Chai et al., 2013).

As mentioned in section 1.5.2, the HDL-raising mechanisms of niacin are complex, but may be partially linked to GPR109A-mediated induction of cholesterol

efflux from macrophages (Lukosova et al., 2011; Rubic et al., 2004). Niacin can target GPR109A to reduce the progression of atherosclerosis by inducing the expression of ABCG1 cholesterol transporter and increased cholesterol efflux to HDL in macrophages (Lukasova et al., 2011).

1.6.2 Endothelial Cells

Several lines of evidence in rodent models exist that support of the concept that niacin can improve endothelial cell function independent of correcting dyslipidemia. In rats, niacin has been shown to increase angiogenesis in areas of brain ischemia, as shown by increased expression of VEGF, ANGPT1, and eNOS, and increased capillary formation (Chen et al., 2007). In diabetic mice, niacin has been shown to enhance revascularization in areas of peripheral ischemia, independent of changes in plasma lipids (Huang et al., 2012). And, in rabbits, treatment with niacin decreased endothelial expression of VCAM-1 and MCP-1 (Wu et al., 2010), showing that niacin inhibits vascular inflammation and protects against endothelial dysfunction.

These findings in rodent models are supported by several human clinical studies. Thoenes et al. (2007) measured endothelial function by using ultrasound to evaluate endothelium-dependent flow-mediated vasodilation of the brachial artery, and found a significant improvement in patients treated with niacin. Improvements in forearm blood flow have also been observed in patients with type 2 diabetes mellitus following niacin (Hamilton et al., 2010). Further evidence linking treatment with niacin to decreased vascular oxidative stress has recently been shown among healthy middle aged and older adults (Kaplon et al., 2014). With regards to a potential mechanism for this effect, niacin has been shown to inhibit ROS production in human aortic endothelial cells (HAEC),

accompanied by a reduction in NF-κB activity and inhibition of vascular inflammation (Ganji et al., 2009; Vosper, 2009). Finally, recent meta-analyses by Sahebkar (2014) confirmed that treatment with niacin improves endothelial function in human populations.

1.7 Endothelial Cell and Mouse Models of Lipotoxicity and Ischemia

1.7.1 Human Microvascular Endothelial Cells

Many studies of EC function are based on experimentation in human umbilical vein endothelial cell (HUVEC). Although much has been learned using this cell type, these cells are derived from vessels which are rarely affected by the most common vascular disorders during metabolic syndrome (Cines et al., 1998). To most closely approximate the cell type which would be involved in revascularization after ischemic injury, human microvascular endothelial cell (HMVEC) from normal skin tissue were chosen for the experiments presented in this thesis. These cells are derived from a vascular bed composed of small vessels which participate in wound healing.

1.7.2 Diet Induced Obese Mice with Peripheral Ischemic Injury

The most commonly used mouse model for studies of the effects of diet-induced obesity is the C57BL/6J mouse. However, this strain carries 2 mutations in the gene encoding nicotinamide nucleotide transhydrogenase (Nnt), which has a role in NAD^+ metabolism and the maintenance of mitochondrial function (Sazanov and Jackson, 1994). Since niacin is a precursor for NAD^+ synthesis (Hara et al., 2007), use of this strain and may have confounded our study. Therefore, for the *in vivo* portion of this thesis, the 129S6/SvEv strain, which does not carry mutations in Nnt, was used. Importantly,

129S6/SvEv mice do become obese, and exhibit many characteristics of metabolic syndrome, after being fed a high fat (western) diet for several months.

1.8 Objectives and Hypothesis

1.8.1 Rationale

Obesity and metabolic syndrome are now epidemic worldwide, and are associated with greatly increased risk for vascular diseases, including peripheral ischemia. Endothelial cells are particularly prone to lipotoxicity, which occurs during obesity and metabolic syndrome, because they are not metabolically programmed to process large amounts of lipid (Helies-Toussaint *et al*, 2006; Staiger *et al* 2006). During peripheral ischemia, endothelial cells mount an angiogenic response to hypoxic conditions in an attempt to restore blood flow (Manalo *et al*, 2005; Luo *et al,* 2012). However, hypoxia also diminishes cellular capacity for β-oxidation, thereby limiting the clearance of excess fatty acids through this metabolic pathway.

Niacin has been used clinically for decades to lower blood triglycerides and free fatty acids, and thereby reduce the risk of vascular disease (Creider *et al*, 2012). However, recent studies indicate that niacin can improve vascular function and vascular regeneration after injury, and that these effects may not be dependent on its systemic lipid lowering abilities (Chai et al., 2013; Digby et al, 2012; Huang et al., 2012; Lukasova et al., 2011; Tavintharan et al., 2011; Wu et al., 2010). Our lab recently showed that supplementation with 10 μM niacin (a low, pharmacologically relevant concentration) improves human microvascular endothelial cell tube formation on Matrigel during exposure to excess palmitate under normoxic conditions (20% O_2). This affect appeared

to be mediated in part by the niacin receptor, GPR109A, which was found to be expressed by these cells (Hughes-Large et al., 2014, submitted). Whether excess fatty acids cause lipotoxicity in endothelial cell during hypoxia, and whether niacin can improve endothelial cell angiogenic function under these conditions, *in vitro* and *in vivo*, is unknown.

1.8.2 Hypothesis

Microvascular endothelial cell angiogenic function during hypoxia will be impaired under conditions of lipid overload, and may be improved by treatment with niacin.

1.8.3 Objectives

The specific objectives of this thesis were to:

1) Determine the effect of excess fatty acids on microvascular endothelial cell survival and angiogenic function under hypoxic conditions *in vitro*.

2) Determine whether niacin can improve microvascular endothelial cell angiogenic function under hypoxic and lipotoxic conditions *in vitro*.

3) Determine whether niacin can improve functional recovery after femoral artery ligation and excision in a mouse model of obesity and metabolic syndrome.

1.8.4 Relevance to Disease

Endothelial dysfunction is an important initial step in the development of vascular complications during obesity and metabolic disease, and the maintenance of endothelial cell function opposes the development of vascular disease (Verma et al., 2003; Xu and Zou, 2009). Recent studies showed niacin to improve vascular function, independent of

systemic lipid lowering. Determining whether niacin can improve human microvascular endothelial cell function during lipotoxicity, and by what mechanism, may be important steps in determining further clinical applications of this drug. The work described in this thesis could have implications for the use of niacin as a treatment for ischemic microvascular complications during metabolic disease.

Chapter 2

2 Methods

2.1 Cell Culture and Treatments

Primary human microvascular endothelial cells (HMVEC) were utilized for all *in vitro* experiments. HMVEC, from normal skin tissue of adult donors, were obtained from Lonza and cultures were maintained in Medium 199 (Invitrogen) supplemented with EGM-2-MV SingleQuots (Lonza), on 100 mm culture dishes, at 37°C and 5% CO_2. Growth medium was changed every 2-3 days, and cells were subcultured at 80% confluence. For all experiments, cells were used between passages 4 and 10.

Fatty acids were used at concentrations of 0.5 mM palmitate, 0.5 mM oleate, or 0.5 mM of a combination of palmitate and oleate (1:1 ratio). Stock solutions of 20mM palmitate or 20 mM oleate were prepared by saponification using 0.1 M NaOH and incubation at 70°C for 30 min. Fatty acid stock solutions were complexed with fatty acid free 30% bovine serum albumin (BSA) (Sigma) at a molar ratio of 2:1 (fatty acid to BSA). Growth medium, supplemented with EGM-2-MV SingleQuots (Lonza), was added to reach a final fatty acid concentration of 0.5 mM (Borradaile et al, 2006). All fatty acid containing experimental media were warmed to 37°C for a minimum of 30 min prior to use, to ensure equilibrium between albumin and fatty acids. Medium, with EGM-2-MV SingleQuots (Lonza), supplemented with BSA alone was used as a control. The concentrations of fatty acids used reflect the high physiological to pathophysiological concentrations observed during metabolic syndrome, obesity and type 2 diabetes mellitus

(Soriguer et al., 2009). The 1:1 ratio of palmitate and oleate is similar to the ratio of saturated and unsaturated fatty acids in typical Western diets (Staiger et al., 2004).

For experimental conditions which included niacin, media were supplemented with 10 μM niacin (Fluka BioChemika). This concentration of niacin reflects average plasma concentrations achieved following pharmaceutical dosing (Menon et al., 2007). For addition to media, niacin was dissolved in cell culture grade water at room temperature. Cell culture grade water was added as a vehicle control where required. Cells were pre-treated for 24 h with niacin, followed by re-addition of niacin during experimental incubations.

For *in vitro* experiments involving hypoxic conditions, oxygen concentrations were lowered to 2% by displacement with nitrogen in a CO_2/O_2 incubator (Panasonic). Temperature and CO_2 were maintained at 37°C and 5%, respectively. Re-oxygenation treatments were achieved by incubating cells for 24 h in hypoxic conditions, followed by a further 24 h in normoxia (20% oxygen).

2.2 Palmitate Oxidation

HMVEC were seeded overnight, in duplicate, at 75,000 cells per well in a 24-well plate. Two wells were left without cells, in order to measure background radioactivity. Prior to the addition of radioisotope, growth medium was removed and cells were washed with warm phosphate buffered saline (PBS). Growth medium containing either DMSO (vehicle control) or 200μM etomoxir, a carnitine palmitoyl transferase I (CPT1) inhibitor, was added, followed by addition of ^{3}H-palmitate complexed to BSA to yield 2.0 μCi/mL ^{3}H-palmitate in 100 μM palmitate. Cells were then incubated for 1 h at 37°C. Culture

plates were subsequently placed on ice and media were removed to individual glass test tubes. Unreacted fatty acids were extracted from media by 4 washes with hexane. Aliquots of the aqueous layers were transferred to scintillation vials for counting. Cell proteins were dissolved in 1 N NaOH, and then neutralized with 1 N HCl for measurement of total cell protein. Counts measured represented the amount of ^{3}H-palmitate oxidized to ^{3}H-H_2O, and were expressed per mg of cell protein. This protocol was modified from Borradaile and Pickering, 2009.

2.3 Cell Viability

A Dead Cell Apoptosis Kit (Invitrogen) was used to assess HMVEC apoptosis and cell death, by staining with Alexa Fluor 488 annexin V and propidium iodide respectively, followed by flow cytometry. Cell staining was performed according to the protocol of the manufacturer (Molecular Probes). Following cell culture incubations, as indicated, cells were washed in cold PBS and harvested with trypsin-EDTA. Cell samples were resuspended in 200 µL annexin V binding buffer at a concentration of approximately 1×10^{6} cells/mL. A working solution of 100µg/mL of propidium iodide was prepared in Annexin V binding buffer. Annexin V and propidium iodide working solution were added to each cell sample. Unstained and single stained (either annexin V or propidium iodide) control cell samples were prepared at the same time, to facilitate the adjustment of flow cytometer settings. Samples were analyzed by flow cytometry (London Regional Flow Cytometry Facility, Robarts Research Institute, Western University). Fluorescence was measured at 530nm and >575 nm using 488 nm excitation. Cell populations were gated on an unstained control and voltages were set with single stained controls. Apoptotic cells were defined as annexin V positive and propidium iodide negative. Late apoptotic and

necrotic cells were defined as both annexin V and propidium iodide positive. Live cells were negative for both annexin V and propidium iodide.

2.4 Growth Rate

HMVEC were grown to 80% confluence in 100mm dishes. HMVEC were harvested with trypsin-EDTA and then were plated at a known density of 2500 cells/cm^2 or 24,000 cells per well in a 6-well plate under various conditions for 48 h, as indicated. Cells were then harvested with trypsin-EDTA and counted with a hemocytometer. Population doublings were calculated using the following equation: $\log_{10}$(number of cells harvested) – $\log_{10}$(number of cells seeded) × $(\log_{10}2)^{-1}$.

2.5 Tube Formation

Prior to assessing tube formation, HMVEC were plated at 80% confluence in a 6-well plate. Cells were pre-treated for 24 h with niacin at varying oxygen levels, as indicated. Matrigel™ Basement Membrane Matrix (BD Biosciences) was thawed on ice overnight at 4°C, and subsequently added to pre-chilled 24-well plates (200 µL per well). The plate was incubated for 30 min at 37°C to solidify the Matrigel before plating the cells. In order to prepare the cells for plating, HMVEC were harvested with EDTA-trypsin and resuspended in fatty acid supplemented media to a concentration of 3.75-5.0 $\times 10^5$ cells/mL, with or without the addition of niacin, as indicated. Prepared cell suspensions were dispensed (200 µL per well) onto Matrigel-coated wells. Plates were incubated for a further 18 h, at the indicated oxygen concentrations, to allow HMVEC capillary tube formation. Resulting tubes were visualized by light microscopy using an Olympus IX71 microscope. For each well, three fields of view were imaged, and total tube length per

field of view was quantified using ImageJ. A tube was defined as a multicellular elongated structure stretching between branch points, and having a width approximately large enough along its entire length to permit the passage of an erythrocyte (~8µm). This protocol was modified from Borradaile and Pickering, 2009.

2.6 siRNA Transfection

Prior to transfection, HMVEC were seeded at 60% confluence on a 6-well plate. A Cy3 labeled control siRNA (Locus ID 338442, Ambion AM6421) or one of three siRNA targeted against GPR109A (Silencer Select Clone IDs s50342, s5033, s50344, Ambion) were transfected into HMVEC using Lipofectamine RNAiMAX (Life Technologies). For preparation of transfection reagents, 3µL of 10 µM siRNAs were diluted in 150 µL of Opti-MEM medium (Life Technologies). Separately, 9 µL of Lipofectamine RNAiMAX reagent were also diluted in Opti-MEM medium. Diluted siRNAs were added to the diluted Lipofectamine RNAiMAX reagent (1:1 ratio) and incubated for 5 min at room temperature. siRNA-lipid complexes were added to the pre-seeded HMVEC cultures, and cells were subsequently incubated for 48 h, followed by treatment for 24 h with or without niacin, as indicated. HMVEC were then harvested and used for tube formation assays, as described in Section 2.5.

Transfection efficiency at 24 h, in cells transfected with control Cy3 labeled siRNA, was estimated to be 80% by fluorescence microscopy. Knockdown of GPR109A protein was confirmed by immunoblotting (as described in Section 2.7) at 72 h. This time point matched the plating of corresponding tube formation assays.

2.7 Immunoblotting

Whole cell lysates were prepared by incubation for 20 min on ice with RIPA buffer (Sigma), supplemented with protease inhibitors (Roche). Lysates were centrifuged for 2 min at 16,000 ×g to eliminate insoluble material. Total cellular protein was determined using a Bicinchonic Acid (BCA) protein assay kit (Thermo Scientific). Depending on the protein of interest, 30-50 µg of total cell protein were diluted with Laemmli sample buffer containing β-mercaptoethanol. Samples were heated to 95°C for 5 minutes. Protein samples were resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a 0.45 µm PVDF membrane using a semi-dry transfer apparatus (Thermo Scientific). Membranes were subsequently incubated in 1% blocking buffer (Roche) for 1 h at room temperature, followed by an overnight incubation at 4°C with a 1:600 dilution of anti-GPR109A rabbit polyclonal antibody (Abcam). After three 5 min washes with Tris buffered saline containing Tween-20, a 1:5000 dilution of horseradish peroxidase (HRP)-conjugated polyclonal anti-rabbit secondary antibody (Santa Cruz Biotechnology) was added for 1 h at room temperature. Membranes were again washed 3 times for 5 min, followed by incubation for 1 min with Enhanced Chemilumnisence Reagent (Roche) and exposure to film. Consistency of protein loading was determined by overnight incubation with a 1:5000 dilution of anti-GAPDH mouse polyclonal antibody (Enzo), followed by a 1 h incubation with 1:5000 dilution of HRP-conjugated polyclonal anti-mouse antibody (Santa Cruz Biotechnology) at room temperature. Bands for GPR109A and GAPDH were detected at 42 and 37 kD respectively, by comparison to Precision Plus Protein All Blue Standards (Biorad). Bands were quantified by densitometry using Quantity One 1-D Analysis Software (Biorad).

2.8 Mice

In order to induce characteristics of metabolic syndrome, five week old male 129S6/SvEv mice (Taconic) were fed western diet containing 42% of calories from animal fat (Harlan Teklad) *ad libitum* for 15 weeks. Control mice were maintained on chow diet with 4% of calories from fat (Harlan Teklad).

After 15 weeks of control or western diet, mice underwent right femoral and saphenous artery ligation, followed by complete excision of the femoral artery. Surgery was done above and below the profunda femoris artery with 6-10 silk sutures. Control and obese mice were randomized into 3 groups for subsequent treatment with vehicle (saline) or niacin. For 14 days following surgery, mice received once daily intra-peritoneal (i.p.) injections of vehicle or niacin (50 mg/kg).

Recovery of hind limb function was assessed on post-surgery days 4, 9, and 15 by gait analyses using a Catwalk system (Noldus) to observe paw contact time and intensity (Frontini et al., 2011). The Catwalk system was located in at the Neurobehavioural Core Facility at Robarts Research Institute, Western University.

At sacrifice (day 16), blood and tissues (liver, adipose) were harvested and tibialis anterior muscles were isolated. Plasma triglycerides and cholesterol were determined by enzymatic, colorimetric assays with reagents from Roche Diagnostics. Blood glucose was determined with an Ascensia Elite glucometer (Bayer) and plasma insulin was measured using a mouse ultrasensitive insulin enzyme-linked immunosorbent assay (ELISA) (Alpco Diagnostics). Plasma liver enzymes (ALT and AST) were measured at the London Health Sciences Centre Core Laboratory. Total weights of liver and adipose were measured, and

liver cholesterol and triglycerides were determined by enzymatic, colorimetric assays (Roche Diagnostics and Wako Diagnostics). All plasma and tissue biochemical measurements, with the exception of plasma ALT and AST, were performed through the Metabolic Phenotyping Laboratory in Robarts Research Institute, Western University.

Tibialis anterior muscles were immersed in zinc fixation buffer and embedded in paraffin. Cross-sections 5µm thick were sliced with a microtome (Leica), and were analyzed after double immunostaining for CD31 and smooth muscle α-actin. The anti-body sources were from rats (LEW) and mouse clones 1A4 respectively, with hematoxylin as a counter stain. Vessel density and smooth muscle α-actin positive vessels were quantified in 12 high powered (x20 objectives) fields at each of three equally spaced sectioning planes of the tissues (van der Veer et al.,2005), using ImageJ. Stained tissue sections were imaged using an inverted Olympus BX51 microscope.

2.9 Statistics

Statistical analyses were performed using either a one-way ANOVA followed by Tukey's post hoc test or a two-way ANOVA followed by a Bonferroni's post hoc test. Differences in means were considered statistically significant when $p < 0.05$. GraphPad Prism version 5.0 software was used for all statistical analyses and generation of graph.

Chapter 3

3 Results

3.1 HMVEC fatty acid oxidation and cell survival are reduced under lipotoxic and hypoxic conditions

During hypoxia, cells limit fatty acid β-oxidation to conserve oxygen (Bhatnagar, 2003). Since human endothelial cells have been shown to use fatty acids, to some degree, to generate ATP (Dagher et al., 2001), we proposed that low oxygen conditions would limit palmitate β-oxidation in HMVEC. We found that HMVEC β-oxidation of palmitate was significantly reduced under hypoxic conditions (2% O_2) (Figure 3.1), confirming functional hypoxia.

It is generally known that saturated fatty acids can induce apoptosis in a dose dependent manner; whereas unsaturated fatty acids do not, and can even stimulate proliferation (Ciapaite et al., 2007; Staiger et al., 2006; Brookheart et al., 2009). Thus, we anticipated that the combination of hypoxia and high palmitate would reduce HMVEC survival. To determine whether hypoxia affects cell survival during fatty acid overload, HMVEC treated for 24 h with fatty acids, in either normoxia or hypoxia, were stained with Annexin V (AnnV), as an indicator of apoptosis, and with propidium iodide (PI), as an indicator of late apoptosis and necrosis (Figure 3.2). Cell staining was analyzed by flow cytometry. To approximate conditions which might occur in vessels during ischemia and reperfusion *in vivo* (i.e. hypoxia followed by re-oxygenation and exposure to circulating lipids), we also performed experiments in which cells were preinucbated for 24 h at 2% O_2, followed by further 24 h incubation at 20% O_2 with the addition of excess fatty acids (Figure 3.2D). HMVEC cell death was increased during palmitate overload

under hypoxic conditions (2% O_2) (Figure 3.2C). Although total cell death was increased during palmitate overload (Figure 3.2B-D), there was no significant reduction in cell survival in hypoxia relative to normoxia and HMVEC survival appears to not be significantly affected by hypoxia alone.

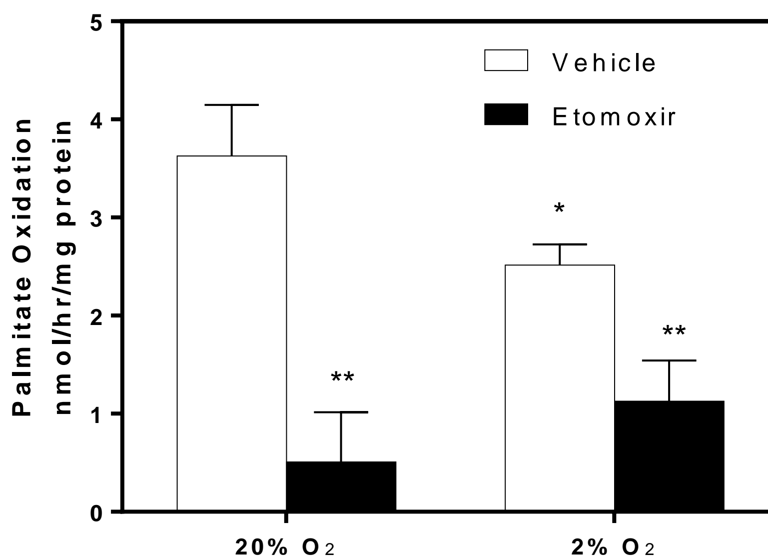


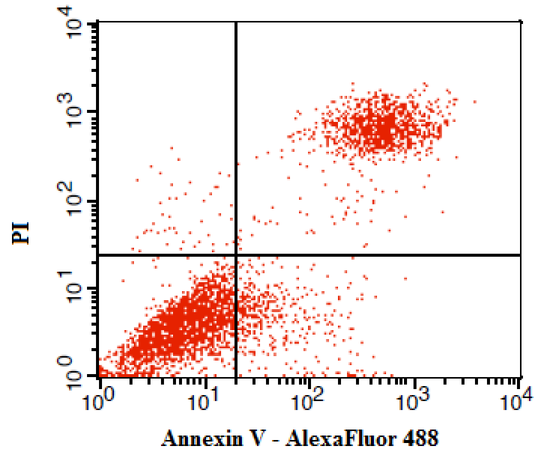
Figure 3.1. HMVEC β-oxidation of palmitate is reduced under hypoxic conditions.
Palmitate β-oxidation was assessed by conversion of ^{3}H-palmitate to water. Cells were pre-incubated in normoxic (20% O_2) or hypoxic (2% O_2) conditions for 24h followed by incubation with labelled palmitate complexed to BSA, for 1 h. Tritium radioactivity in media and aqueous cell fractions were determined by liquid scintillation counting. Etomoxir (200 μM), a CPT1 inhibitor, was used as a negative control. Data are expressed as means ± SEM for 5 independent experiments. * $p < 0.05$ vs. vehicle 20% O_2. **$p<0.05$ vs. respective vehicle control.

Figure 3.2. HMVEC cell death is increased during palmitate overload under hypoxic conditions.

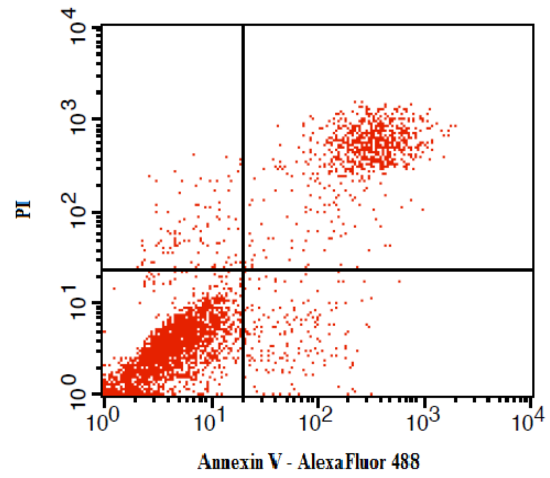
Cells were incubated for 24h with medium containing BSA (control), 0.5 mM palmitate (PA), 0.5 mM oleate (OA), or PA + OA (1:1, 0.5 mM), all complexed to BSA (molar ratio 2:1). Cell survival was assessed by staining with annexin V (AnnV) and propidium iodide (PI), with quantification by flow cytometry. Representative dot plots for HMVEC in hypoxia (2% O_2) are shown **(A)**. Percentages of dead cells ($AnnV^+/PI^+$) and apoptotic cells ($AnnV^+$) were determined under normoxia (20% O_2) **(B)**, hypoxia (2% O_2) **(C)**, and conditions representative of hypoxia followed by re-oxygenation (24h preincucation at 2% O_2, followed by 24h incubation at 20% O_2 in presence of fatty acids; 2% O_2 → 20% O_2) **(D)**. Data are expressed as means ± SEM for 5 independent experiments. * $p < 0.05$ vs. BSA.

A

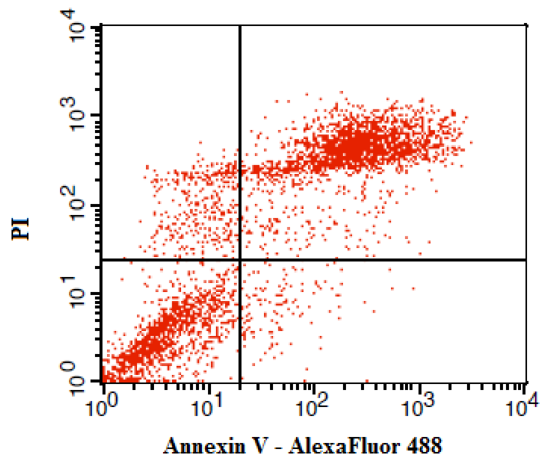
BSA



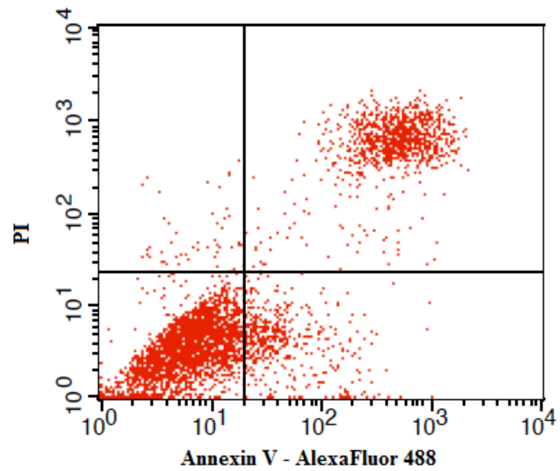
OA



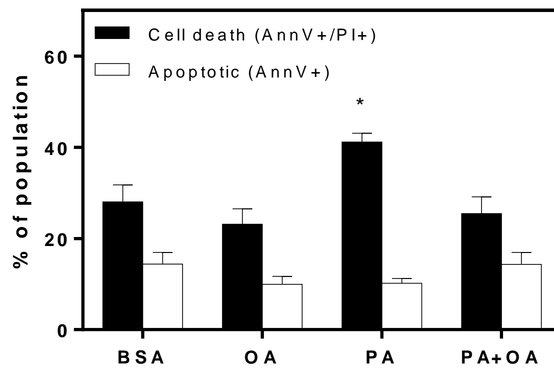
PA



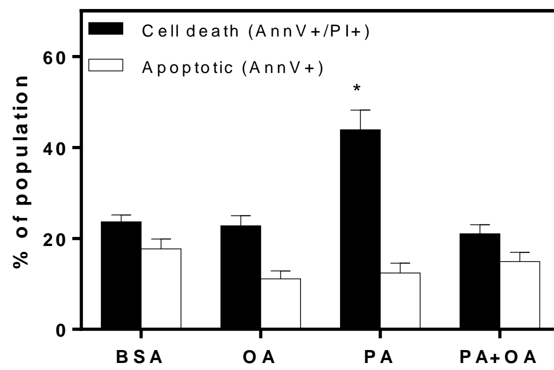
PA+OA



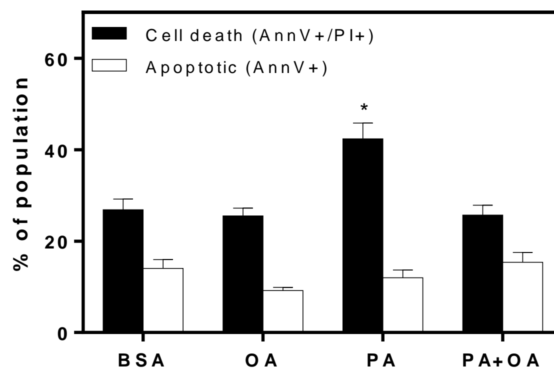
B 20% O_2



C 2% O_2



D 2% → 20% O_2



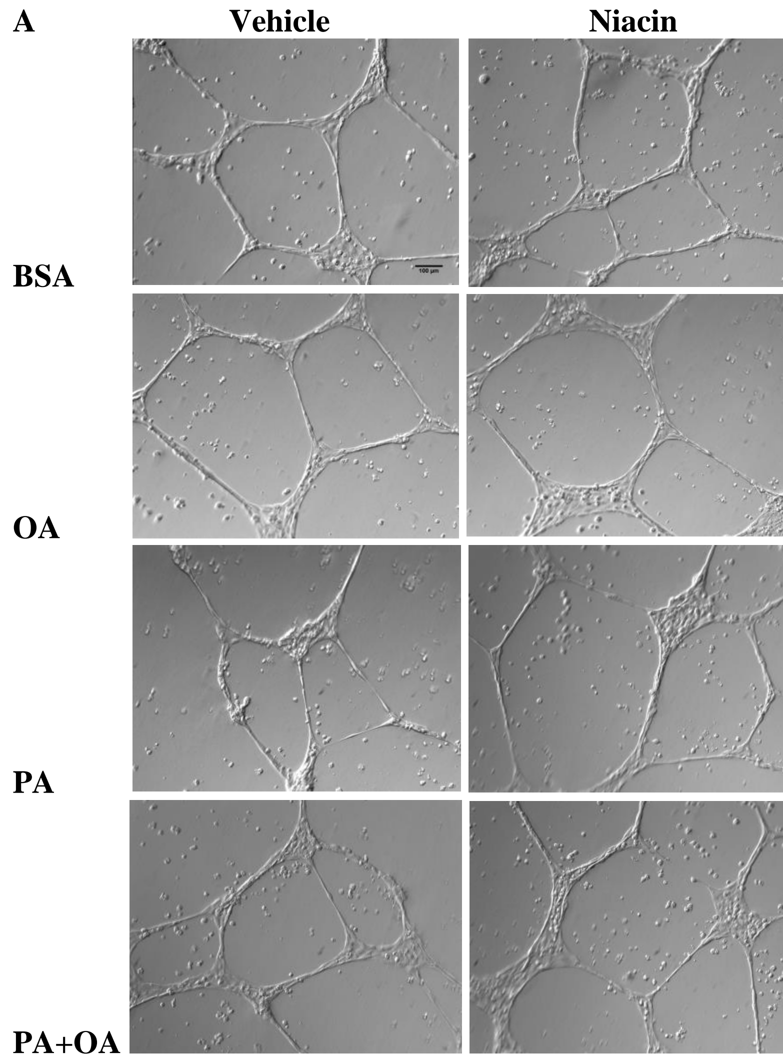
3.2 HMVEC angiogenic function is improved by niacin under lipotoxic and hypoxic conditions

Niacin has been used clinically for decades to lower triglycerides (Kamanna et al., 2013). However, niacin has also recently been reported to improve endothelial cell function independent of correcting dyslipidemia (Huang et al., 2012; Thoenes et al., 2007), and to increase angiogenesis in ischemic brains of adult male rats (Chen et al., 2007). Thus, we determined whether niacin would improve HMVEC angiogenic function during fatty acid overload and hypoxia, conditions which would be observed in peripheral ischemia during metabolic syndrome.

In normoxia, HMVEC exhibited impaired tube formation on Matrigel after 18 h of treatment with growth medium containing saturated fatty acid palmitate, whereas treatments with unsaturated fatty acid oleate or combination of palmitate and oleate did not significantly impair tube formation (Figures 3.3). Niacin improved HMVEC tube formation during fatty acid overload under normoxic conditions (Figure 3.3). Under low oxygen (2% O_2) conditions, tube formation was reduced for all control and fatty acid treatments (Figure 3.4 vs. Figure 3.3). However, niacin was also able to improve tube formation under hypoxic conditions for all control and fatty acid treatments (Figure 3.4). In order to approximate ischemic insult followed by re-oxygenation, cells were incubated for 24 h in hypoxic conditions, followed by 24 h in normoxia. Niacin was not able to significantly improve HMVEC tube formation under these conditions (Figure 3.5).

Figure 3.3. Niacin improves HMVEC tube formation during fatty acid overload under normoxic conditions.

Cells pre-treated for 24 h at 20% O_2 in culture medium supplemented with either vehicle (water) or 10 µM niacin (NA) were seeded onto growth factor replete Matrigel and incubated with media containing BSA, 0.5 mM oleate (OA), 0.5 mM palmitate (PA), or palmitate + oleate (PA+OA, 1:1 ratio, 0.5 mM) complexed to BSA. Cells were again treated with either vehicle or 10 µM niacin, and resulting tube networks were analyzed by light microscopy after 18 h incubations at 20% O_2 **(A)**. Scale bar represents 100 µm. For quantification, total tube lengths were measured in three random fields of view per condition **(B)**. Data are expressed as means ± SEM for 5 independent experiments.
* $p < 0.05$ vs. Vehicle, BSA, ** $p < 0.05$ vs. Vehicle, PA.


A
Vehicle
Niacin
BSA
OA
PA
PA+OA

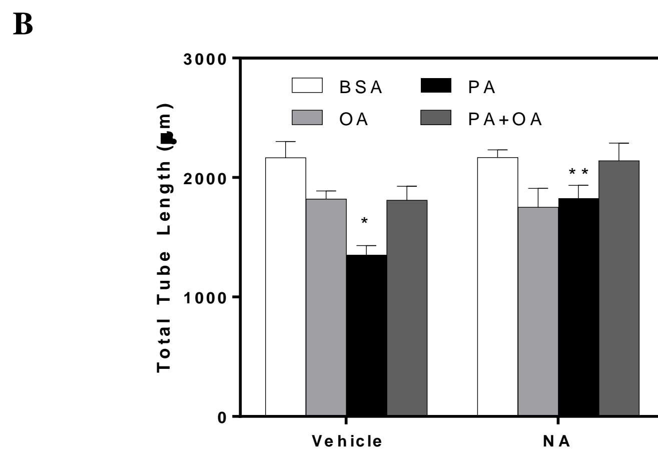
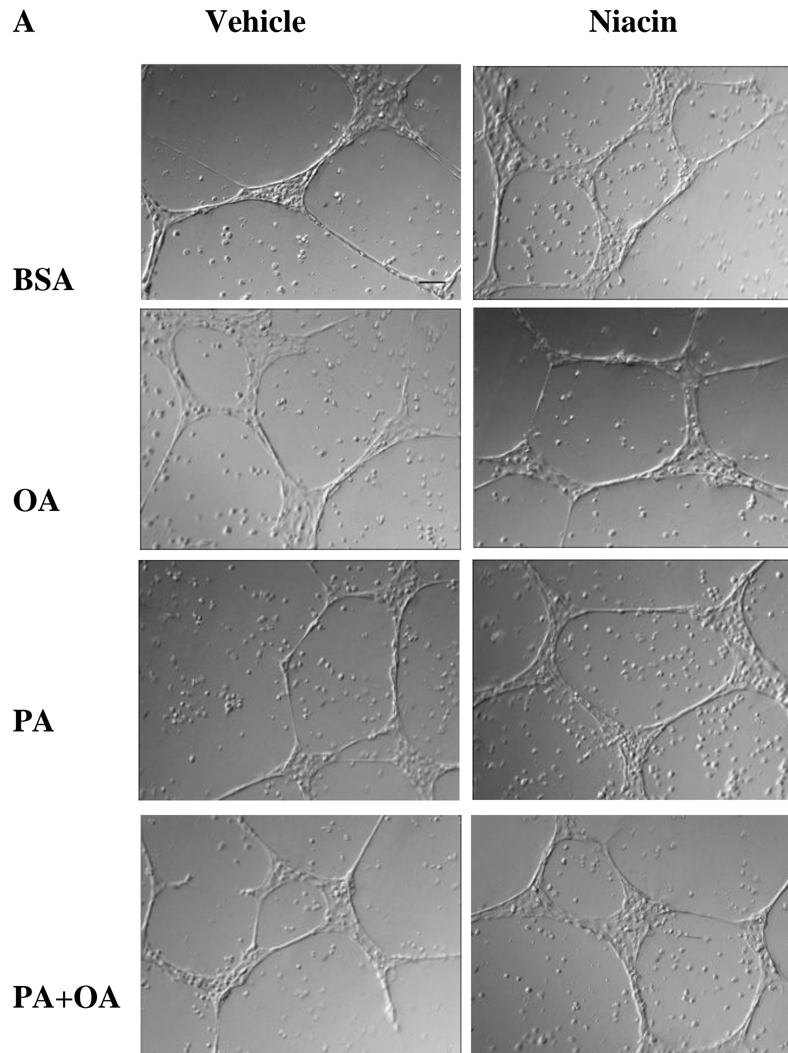
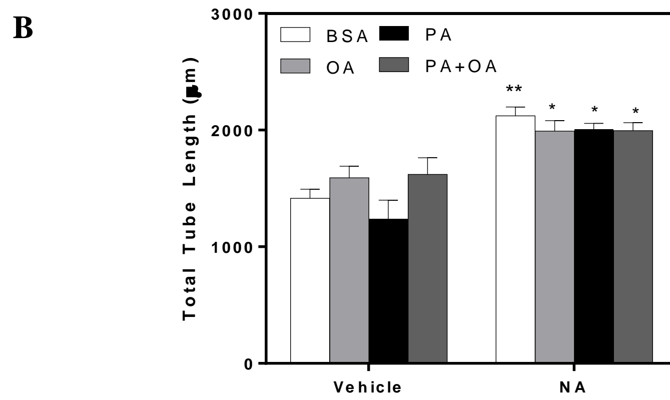

B
3000
2000
1000
0
BSA
OA
PA
PA+OA
Total Tube Length (μm)
*
**
Vehicle
NA

Figure 3.4. Niacin improves HMVEC tube formation during fatty acid overload under hypoxic conditions.

Cells were pre-treated and seeded on Matrigel, in the absence or presence of 10 μM niacin, as described in Figure 3, but under hypoxic conditions (2% O_2). Resulting tube networks were analyzed by light microscopy after 18 h incubations at 2% O_2 **(A)**. Scale bar represents 100 μm. For quantification, total tube lengths were measured for three random fields of view per condition **(B)**. Data are expressed as means ± SEM for 5 independent experiments. * $p < 0.05$ vs. vehicle, BSA, **$p<0.001$ vs vehicle, BSA.


A
Vehicle
Niacin
BSA
OA
PA
PA+OA


B
3000
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Total Tube Length (μm)
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OA
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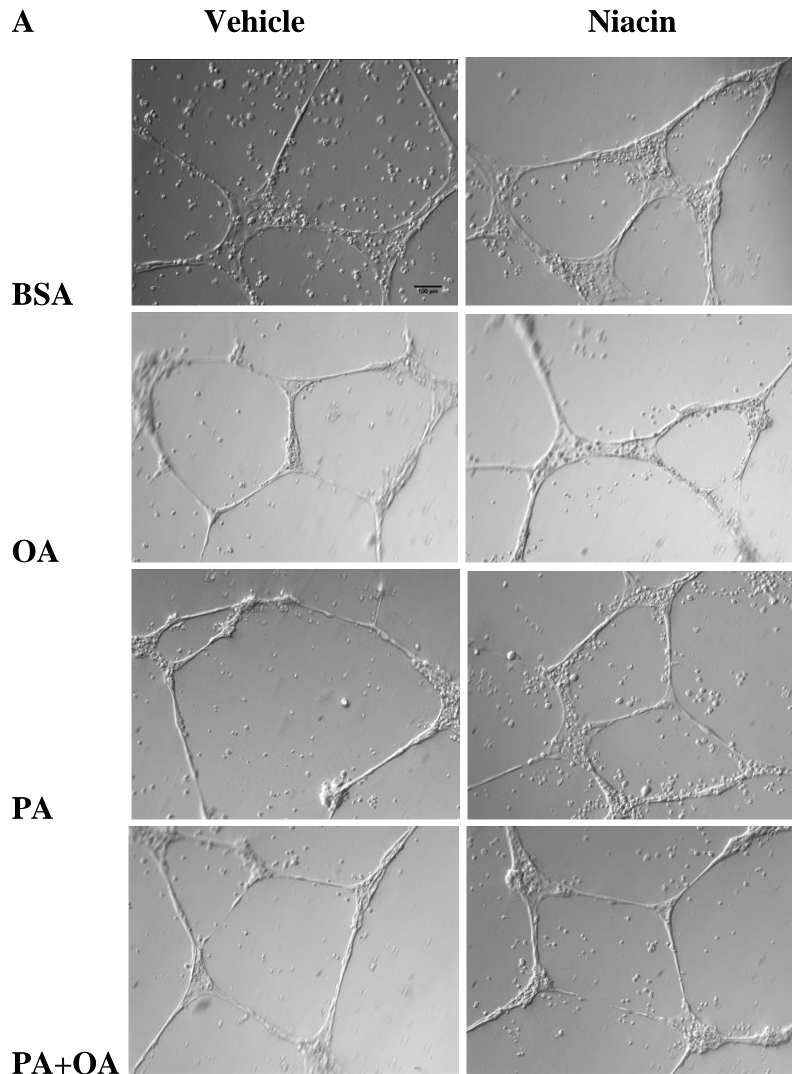
*
Vehicle
NA

Figure 3.5. Niacin does not improve HMVEC tube formation during fatty acid overload under conditions representative of hypoxia followed by re-oxygenation. Cells were pre-treated and seeded on Matrigel, in the absence or presence of 10 μM niacin, as described in Figure 3, but under hypoxic conditions (2% O_2). Resulting tube networks were analyzed by light microscopy after 18 h incubations at 20% O_2 (**A**). Scale bar represents 100 μm. For quantification, total tube lengths were measured in three random fields of view per condition (**B**). Data are expressed as means ± SEM for 5 independent experiments.



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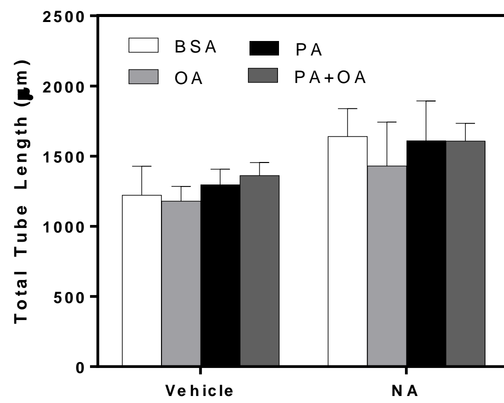


3.3 Partial knockdown of GPR109A diminishes niacin induced improvements in tube formation under lipotoxic and hypoxic conditions

The niacin receptor, GPR109A, has been shown to be expressed in several different tissues and cell types including, adipocytes, intestinal epithelium, epidermal Langerhans cells, and several immune cell types (Ahmed et al., 2009). However, its expression has never been reported in endothelial cells. Whether the effects of niacin on angiogenic function and revascularization involve activation of this receptor on endothelial cells is unknown.

To begin investigating the mechanism for improved HMVEC tube formation with niacin, the effects of niacin on cell proliferation in normoxia and hypoxia were determined. The *in vitro* tube-forming capacity of endothelial cells is a dynamic process that can initially involve cell proliferation. However, as shown in Figure 3.6, basal proliferation rates are unaltered with niacin treatment under normoxic, hypoxic, or re-oxygenation conditions. Furthermore, it was previously shown that niacin does not improve cell survival during fatty acid overload (Hughes-Large et al., 2014, submitted), and that hypoxia does not further increase cell death under lipotoxic conditions (Figure 3.2). Taken together, these data suggest that niacin does not likely act by stimulating cell proliferation or cell survival pathways under lipotoxic and hypoxic conditions.

Our lab has recently shown that HMVEC express GPR109A (Hughes-Large et al., 2014, submitted). In the same study, it was also demonstrated that treating HMVEC with selective GPR109A agonists, acifran and MK-1903, reproduced the effects of niacin on tube formation. To further determine the potential role of the niacin receptor, we used

siRNA to knockdown GPR109A. Relative GPR109A levels were assessed by immunoblotting after 72 h incubation (Figure 3.7). GPR109A siRNA 2 exhibited the most effective knockdown and was used for all subsequent experiments. After transfection with siRNA, HMVEC were seeded onto Matrigel for 18 h to assess tube formation under various conditions (Figures 3.8 and 3.9). Partial knockdown of GPR109A diminished niacin induced improvements in tube formation in high palmitate (Figure 3.8). Similar, but not statistically significant, results were obtained in hypoxia (Figure 3.9). Tube formation, in general, was much lower in transfected cells under hypoxic conditions.

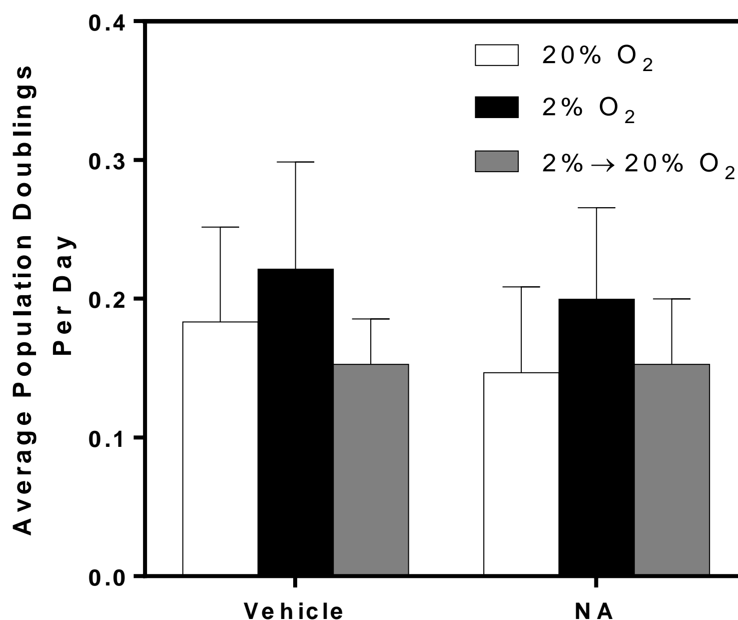


Figure 3.6. HMVEC growth rate is unaffected by supplementation with niacin.

HMVEC were plated at a known density of 2500 cells/cm^2 in medium containing either water (vehicle) or 10µM niacin. Incubation conditions were representative of normoxia (20% O_2), hypoxia (2% O_2), and re-oxygenation (2% → 20% O_2), as described in tube formation assays. Cells were counted and population doublings per day were calculated. Data are expressed as means ± SEM for 5 independent experiments

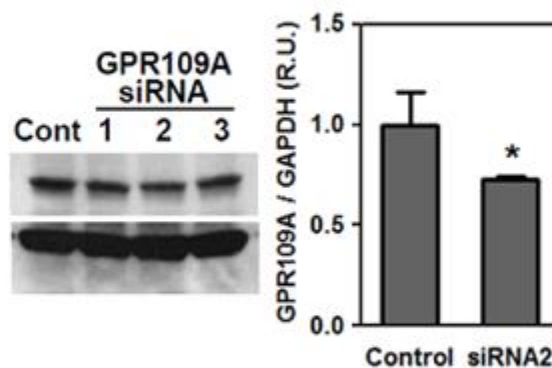
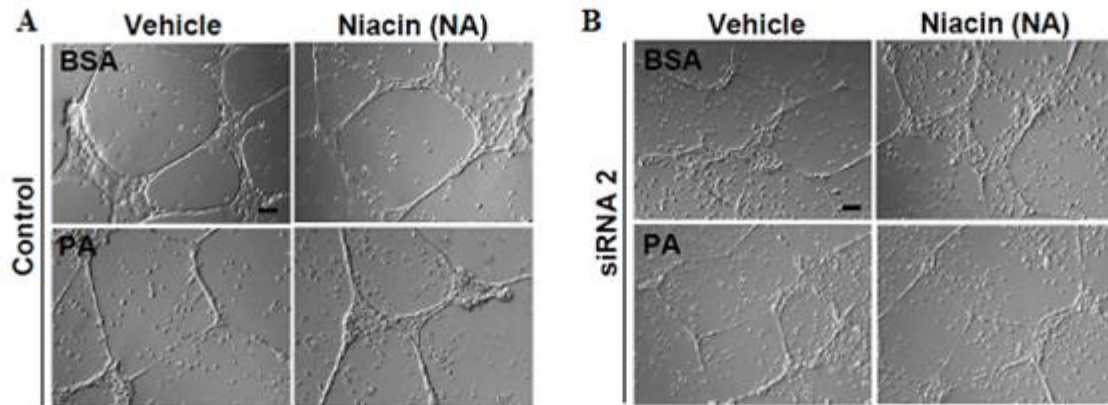


Figure 3.7. Targeted knockdown of GPR109A.

Relative GPR109A levels were assessed by immunoblotting after 72 h incubation with control siRNA (Cont), or one of 3 distinct siRNA targeting GPR109A (1, 2, or 3). Upon establishing that siRNA2 was most effective, densitometry values were determined for GPR109A relative to GAPDH in cells transfected with Control versus siRNA2. Data are means ± SEM for 4 independent experiments. * $p < 0.05$.

Figure 3.8. Partial knockdown of GPR109A diminishes niacin induced improvements in tube formation in high palmitate, under normoxic condtions.

Cells were transfected for 72 h with siRNA against either no known gene sequence (control) **(A)** or GPR109A (siRNA 2) **(B)**, followed by treatment for 24 h with vehicle or 10 μM niacin. Cells were then seeded onto Matrigel in medium containing BSA or 0.5 mM palmitate, and supplemented again with either vehicle or 10 μM niacin. Resulting tube networks were imaged and quantified as described in Figure 3.3 **(C)**. Scale bar represents 100 μm. Data are means ± SEM for 3-4 independent experiments. * $p < 0.05$.


A
Vehicle
Niacin (NA)
BSA
PA
Control
B
Vehicle
Niacin (NA)
BSA
PA
siRNA 2

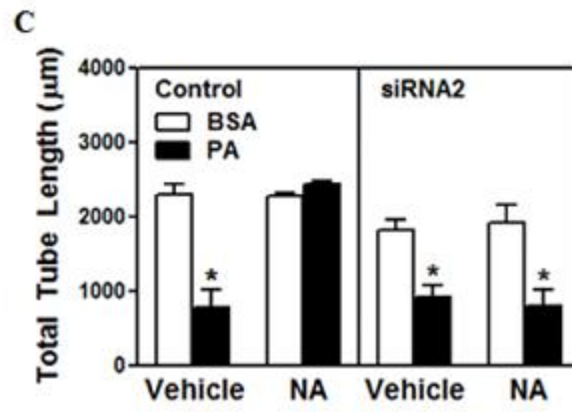
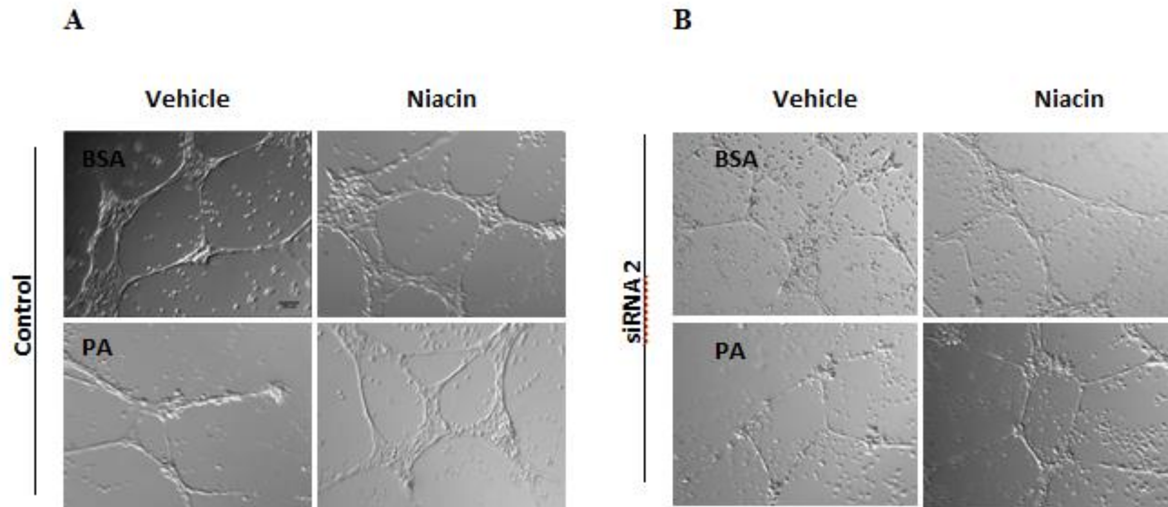

C
Total Tube Length (μm)
4000
3000
2000
1000
0
Control
siRNA2
BSA
PA
*
*
*
Vehicle
NA
Vehicle
NA

Figure 3.9. Effect of partial knockdown of GPR109A on niacin induced improvements in tube formation in high palmitate, under hypoxic conditions.

Cells were transfected for 72 h with siRNA against either no known gene sequence (control) **(A)** or GPR109A (siRNA 2) **(B)**, followed by treatment for 24 h with vehicle or 10 µM niacin. Cells were seeded onto Matrigel in medium containing BSA or 0.5 mM palmitate, and supplemented again with either vehicle or 10 µM niacin under hypoxic conditions (2% O_2). Resulting tube networks were imaged and quantified as in Figure 3.3. Scale bar represents 100 µm. Data are means ± SEM for 3 independent experiments. * $p < 0.05$ and ** $p<0.01$ vs Vehicle BSA.



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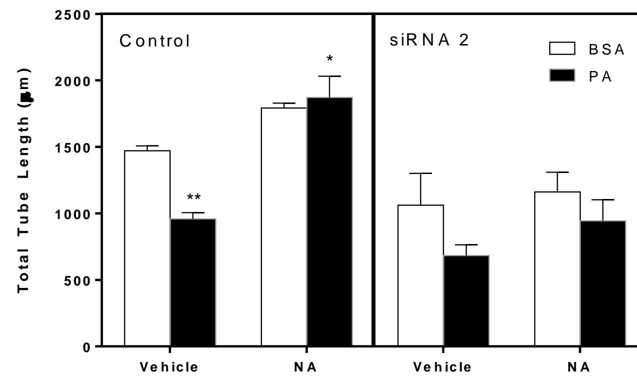


3.4 Niacin improves recovery of hind limb function after ischemic injury in obese mice with metabolic disease

The endothelial cell injury and death that occur during hyperlipidemia can limit the vascular repair and regeneration (Kim et al., 2012). To assess vascular regeneration during metabolic disease *in vivo*, we used 129S6/SvEv mice with diet induced obesity. After 15 weeks of ad libitum feeding of western diet, containing 42% of calories from fat, 129S6/SvEv mice exhibited characteristics of metabolic syndrome, including increased adiposity, hypertriglyceridemia, elevated fasting blood glucose, and hyperinsulinemia (Table 1).

129S6/SvEv mice underwent right femoral artery ligation and excision, followed by 14 days of i.p. injections of either vehicle (water) or niacin (50mg/kg). To determine functional recovery from hind limb injury, a Catwalk System was used to measure paw contact time and intensity during walking on days 4, 9, and 15 after surgery (Figure 3.10). Mean paw contact time ratios and contact intensity ratios over these time points are shown in Figure 3.10B and C. Mean paw contact time on the day prior to sacrifice was significantly increased in mice treated with niacin (Figure 3.10A, D). Mean paw intensity for the same day did not show a statistically significant increase following treatment with niacin, but a trend was observed (Figure 3.10E). These data suggest that treatment with niacin, after ischemic injury, improves recovery of hind limb function in a mouse model of metabolic syndrome.

Table 1. Characteristics of metabolic syndrome in 129S6/SvEv mice

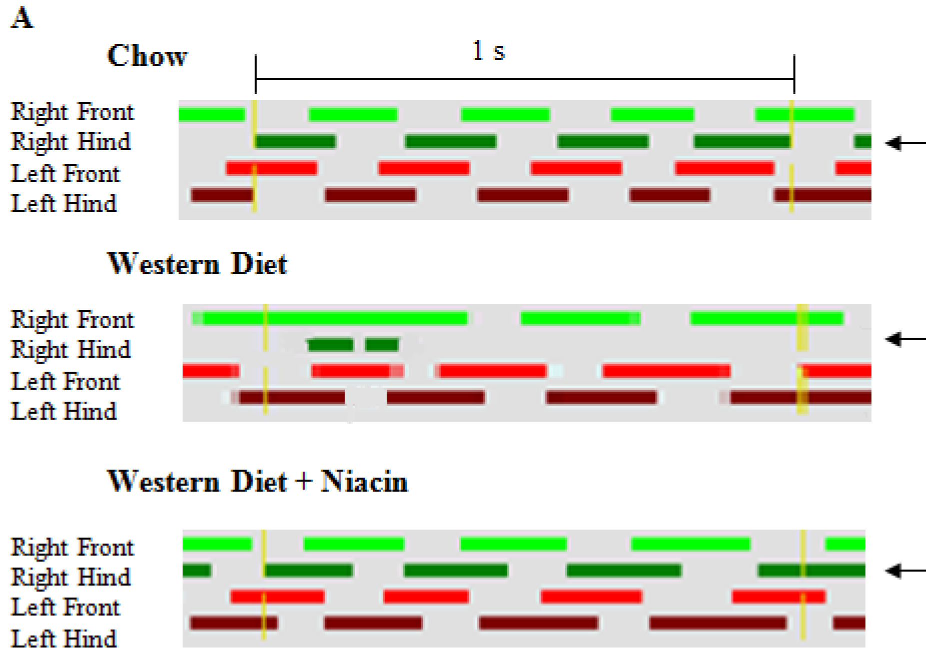
Five week old male mice were maintained on chow (4% calories from fat) or western diet (42% calories from fat) for 15 weeks, followed by unilateral femoral ligation and excision surgery. Mice were maintained for a further 14 days with daily i.p. injections of either sterile water (vehicle) or niacin (50 mg/kg). Body weight and blood glucose measurements were performed immediately prior to sacrifice (day 15). All remaining parameters were determined post-mortem. Data are means ± SEM. Values with different lower case letters are significantly different at $p<0.05$, $n=6$.

Diet (Treatment)	Chow (Vehicle)	Western (Vehicle)	Western (Niacin)
Body weight (g)	26.0 ± 0.7[a]	32.7 ± 1.3[b]	31.1 ± 0.5[b]
Epidydimal fat weight (g)	0.365 ± 0.036[a]	1.442 ± 0.091[b]	1.032 ± 0.114[c]
Liver weight (g)	1.011 ± 0.044	1.107 ± 0.052	1.102 ± 0.044
Liver triglycerides (mg/g)	13.3 ± 2.0[a]	168.4 ± 12.6[b]	66.9 ± 13.3[c]
Plasma ALT (U/L)	35 ± 9.3	46.7 ± 9.3	20.3 ± 1.3
Plasma AST (U/L)	106 ± 16.6	119 ± 19.9	91.2 ± 14.4
Plasma triglycerides (mmol/L)	0.82 ± 0.04[ab]	0.98 ± 0.05[ac]	0.77 ± 0.06 [bd]
Plasma free fatty acids (mmol/L)	0.34 ± 0.02	0.30 ± 0.02	0.26 ± 0.02
Plasma cholesterol (mmol/L)	2.85 ± 0.12[a]	5.24 ± 0.21[b]	3.88 ± 0.09[c]
Blood glucose (mmol/L)	5.2 ± 0.2[a]	6.3 ± 0.1[b]	5.4 ± 0.4[ab]
Insulin (ng/ml)	0.37 ± 0.06[a]	0.74 ± 0.04[b]	0.47 ± 0.09[a]

Figure 3.10. Niacin improves recovery of hind limb function after ischemic injury in obese mice with metabolic disease.

Five week old male mice were maintained on chow or western diet (WD) or chow for 15 weeks, followed by right hind limb femoral ligation and excision surgery. Mice were maintained for a further 14 days on their respective diets with daily i.p. injections of either sterile water (vehicle) or niacin (NA, 50 mg/kg). On days 4, 9, and 15 post-surgery, gait analyses were performed using a Catwalk system. As mice traversed an illuminated glass walkway, the duration (s) of surface contact for each paw was digitally recorded and illustrated as contact duration maps. Representative maps over a 1.0 s time frame are shown for day 15. Arrows indicate the injured limb. **(A)**. Mean paw contact times **(B)** for each hind limb and contact intensities **(C)** (3 replicate walks per mouse per day) were used to calculate hind limb functional recovery. Normal contact time **(B)** and intensity ratio **(C)** over time between hind limbs is approximately 1.0. A right limb to left limb ratio < 1.0 indicates decreased use of the right hind limb. Contact time **(D)** and intensity **(E)** for each experimental group are shown for Day 15. Data are means ± SEM for 6 mice, *p<0.05 vs WD + NA.

A



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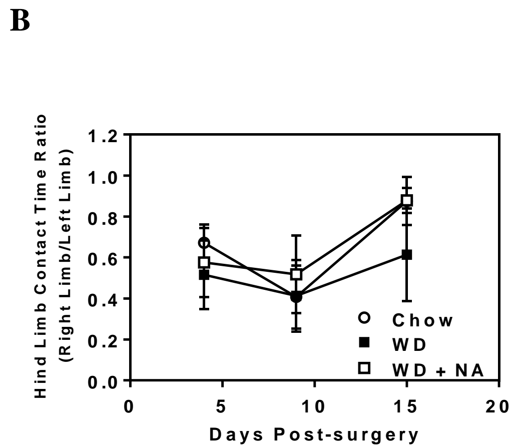

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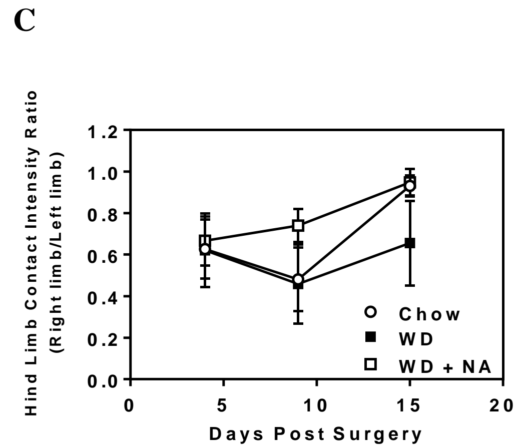
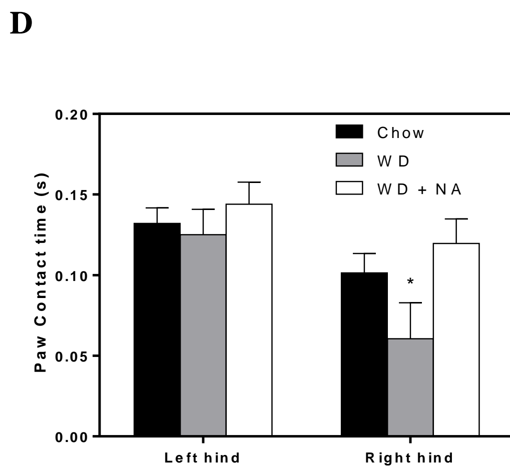


D



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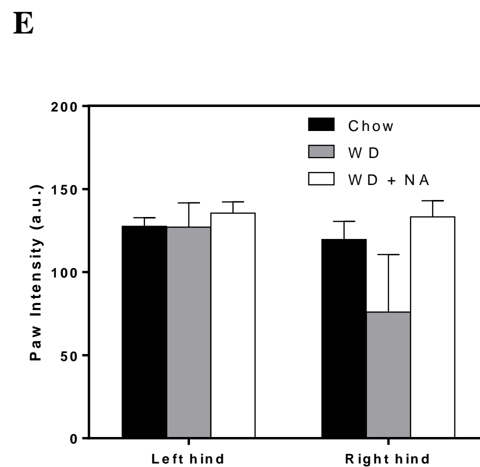

Chapter 4

4 Discussion

4.1 Summary of Results

Vascular complications associated with obesity and metabolic disease result in a large proportion of the morbidity and mortality that accompanies excessive weight gain. Endothelial cell dysfunction precedes the development of vascular diseases such as peripheral ischemia (Campia et al., 2012). Thus, there is interest in determining whether improving endothelial cell function can improve vascular disease outcomes during obesity. Niacin has been reported to have vascular benefits that are independent of its role in improving clinical lipid profiles (Chai et al., 2013). However, whether niacin can improve endothelial cell angiogenic function during saturated fatty acid overload in ischemic conditions, as are observed in the vasculature during obesity, and by what mechanism this might occur, is unknown. For this thesis, it was hypothesized that microvascular endothelial cell angiogenic function during hypoxia would be impaired under conditions of lipid overload, and would be improved by treatment with niacin. Although we cannot explicitly exclude the involvement of the lower affinity niacin receptors, GRP109B and GPER1, the data presented here suggest that niacin may mediate pro-angiogenic effects on endothelial cells through activation of GPR109A.

The major findings of this thesis are as follows: 1) HMVEC fatty acid oxidation and cell survival is reduced under lipotoxic and hypoxic conditions; 2) niacin improves HMVEC tube formation under lipotoxic and hypoxic conditions; 3) HMVEC express the niacin receptor, GPR109A, and partial knockdown of GPR109A with siRNA diminishes

the effects of niacin on tube formation in high palmitate; 4) niacin improves recovery of hind limb function after ischemic injury in obese mice with metabolic disease.

4.2 Niacin and Endothelial Cell Angiogenic Function in Hypoxia

A growing number of reports suggest that niacin can improve vascular function directly, independent of its systemic lipid modifying activity, by modulating the activity of immune cells and endothelial cells (Chai et al., 2013). In support of this concept, studies in rodent models of vascular injury suggest that niacin directly improves vascular endothelial function and vascular regeneration (Chen et al., 2007; Huang et al., 2012; Wu et al., 2010). Improvements in forearm blood flow have also been observed in patients with type 2 diabetes mellitus following niacin (Hamilton et al., 2010). Therefore, we determined whether niacin would have similar benefits in endothelial cells during fatty acid overload and hypoxia. Endothelial cells would be exposed to these conditions during ischemia vascular disease associated with obesity and metabolic syndrome.

In response to hypoxia, endothelial cells mount an angiogenic response to low oxygen conditions in an attempt to restore blood flow (Manalo *et al*, 2005; Luo *et al,* 2012). Interestingly, our *in vitro* data suggest that the combination of high fatty acids and hypoxia generally impairs tube formation. If this data translates to the human *in vivo* scenario, it may suggest that obese patients with peripheral ischemia may not recover blood flow without intervention. Consistent with our previous results (Hughes-Large et al., 2014, submitted), niacin is able to rescue HMVEC tube formation under lipotoxic and hypoxic conditions. These observations of enhanced angiogenic function with niacin,

made under conditions of fatty acid excess and low oxygen, may have implications for the use of niacin in obese or metabolic syndrome patients with ischemic vascular disease.

4.3 Activation of GPR109A and Endothelial Cell Angiogenic Function

Niacin can activate its receptor GPR109A, and this activation has been shown to exert beneficial anti-inflammatory and antiatherogenic effects (Digby et al., 2012; Lukasova et al., 2011). These effects are largely mediated through activation of the receptor in monocytes and macrophages.

Our recent evidence of GPR109A expression in cultured human primary endothelial cells, and in human aortic endothelial cells in situ, was the first of its kind (Hughes-Large et al., 2014, submitted). The improved tube formation elicited by exposure of HMVEC to either niacin or the selective agonists, acifran and MK-1903, in high palmitate, further supported the concept that activation of GPR109A may have beneficial effects on cells of the vasculature (Hughes-Large et al., 2014, submitted). To further investigate the potential role of GPR109A, siRNA was used to knockdown the receptor. Partial knockdown of GPR109A diminished niacin-induced improvements in tube formation in high palmitate. Interestingly, the efficiency of GPR109A siRNA in inhibiting the ability of niacin to improve tube formation was greater than might be predicted based on the amount of knockdown detected at the protein level. There are several possible explanations for this observation. First, it is possible that the GPR109A antibody used is not entirely specific, and may also detect GPR109B. This would result in underestimation of the degree of protein knockdown. However, limited signal (by western blot) was obtained with this antibody in HepG2 cells, which appear to express

more GPR109B than GPR109A (Hughes-Large et al., 2014, submitted). Second, GPR109A siRNA modestly impaired tube formation under basal conditions compared to control siRNA. When this baseline observation is taken into account, the effect of GPR109A siRNA is less dramatic. Third, it is possible that partial impairment of GPR109A signaling is sufficient to block the pro-angiogenic effect of niacin in HMVEC. Although the involvement of the lower affinity niacin receptors, GPR109B and GPER1, cannot be explicitly excluded, the data presented in this thesis suggest that niacin may mediate pro-angiogenic effects on endothelial cells under conditions of metabolic stress through activation of GPR109A.

4.4 Niacin and Functional Recovery in Obese Mice with Acute Ischemic Injury

Data presented in this thesis show that treatment with niacin improved recovery of limb function in diet-induced obese mice with hind limb ischemic injury, as assessed using a Catwalk system immediately prior to sacrifice (Day 15). In fact, animals treated with niacin may exhibit an improved rate of recovery of limb function (Figure 3.10C).

To determine whether improved hind limb function was related to revascularization or improved tissue architecture in the injured muscle, tibialis anterior muscles were harvested at the time of sacrifice, fixed, and stained for markers of endothelial cells (CD31) and smooth muscle cells (smooth muscle α-actin). At the time of writing of this thesis, tissues from only 3 of the 6 mice per experimental group were processed and analyzed. Initial measures of tissue vessel density and vessel smooth muscle investment were inconclusive (Figure 3.11), but analyses are ongoing. Interestingly, muscle bundles in mice maintained on western diet and treated with niacin

appeared to be generally larger than those of control mice. Further analyses will require H&E staining of muscle sections, followed by counting of regenerating (central nuclei) versus necrotic (absent nuclei) myocytes, to determine whether niacin improved muscle tissue regeneration in these mice.

4.5 Future Directions

The *in vivo* work in thesis cannot distinguish between niacin receptor-dependent and –independent effects on revascularization and recovery of hind limb function. To directly implicate GPR109A in any observed effects of niacin on vascular regeneration, hind limb ischemic injury experiments could be conducted in GPR109$^{-/-}$ mice and corresponding wild type littermate controls. These mice are commercially available as cryopreserved embryos on a 129S6/SvEv background (Taconic), and are known to be susceptible to diet induced obesity (Wanders, 2012). Post-surgery treatments with niacin or GPR109A agonists, followed by analyses of functional recovery, metabolic parameters, and tissue architecture would be performed as described in Methods section 2.8. Although the niacin receptor activation has not been studied in endothelial cells, activation of GPR109A-associated pathways in other cell types have been linked to improved angiogenic potential. Prostaglandin E2, a product of GPR109A-mediated cyclooxygenase activation in Langerhans cells of the skin, has been shown to induce angiogenic properties in HMVEC (Salecedo et al., 2003). This could possibly contribute to some of the observed effects of receptor activation, and could also be investigated further in GPR109A$^{-/-}$ mice.

As mentioned in earlier sections of this thesis, a growing number of reports suggest that niacin can improve vascular function independent of its systemic lipid

modifying activity by modulating the activity of immune cells (Chai et al., 2013). Niacin treatment in our model resulted in improved systemic metabolic parameters including plasma lipids (Table 1). Therefore, the lipid-lowering effect of niacin cannot be excluded as a potential part of the mechanism for improved functional recovery in these mice. In order to further address this issue, a time course study could be performed to determine whether functional recovery occurs in niacin-treated mice, prior to any observed reduction in plasma lipids. The possibility that niacin improved inflammation in our model, as has been suggested in previous studies (Chai et al., 2013), will be addressed through ongoing analyses of tissue sections that have already been obtained. Sections of tibialis anterior muscles will be stained for the presence of macrophages.

Finally, many human vascular diseases are restricted to specific types of vessels (Cines et al., 1998). It would be interesting to expand these findings of this thesis to other vascular EC types. One focus of this future direction could be to determine whether GPR109A is expressed on other human endothelial cells types, and to determine the status of niacin receptor expression in different disease states in a variety of vascular beds.

In light of the major findings of this thesis, and the future directions outlined in this section, there may be implications for the use of niacin, or a specific activator of GPR109A, to improve vascular function in the setting of ischemic vascular complications associated with metabolic syndrome.

Figure 3.11. Vessel density of the tibialis anterior muscle of injured right hind limb after ischemic injury in obese mice with metabolic disease.

Five week old male mice were maintained on either chow or western diet (WD), subjected to right hind limb femoral artery excision surgery, and treated for a further 14 days with i.p. injections of either sterile water (vehicle) or niacin (NA, 50 mg/kg). Photomicrographs were taken of sections of the right tibialis anterior muscles. Zinc-fixed sections were immunostained for CD31 (brown, black arrows), to identify endothelial cells, and smooth muscle α-actin (red, white arrows), to identify vascular smooth muscle cells **(A)**. Scale bar represents 100 μm. For quantification, vessel density **(B)** and vessel smooth muscle investment **(C)** were measured in 12 random fields of view per muscle. Data are means ± SEM for 3 mice.

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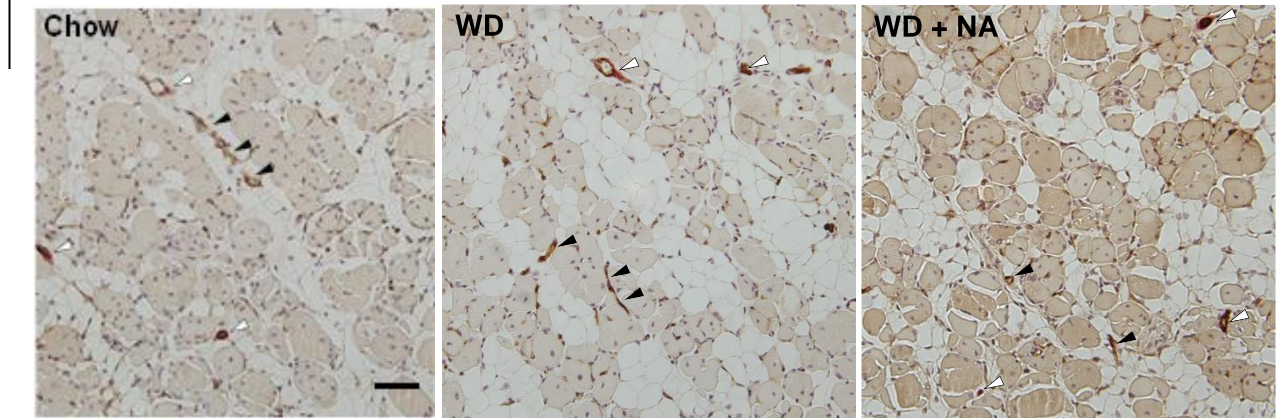

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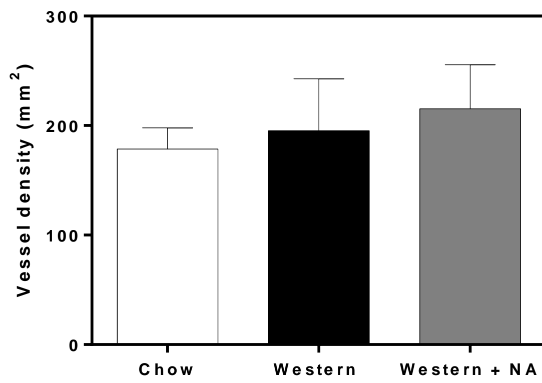

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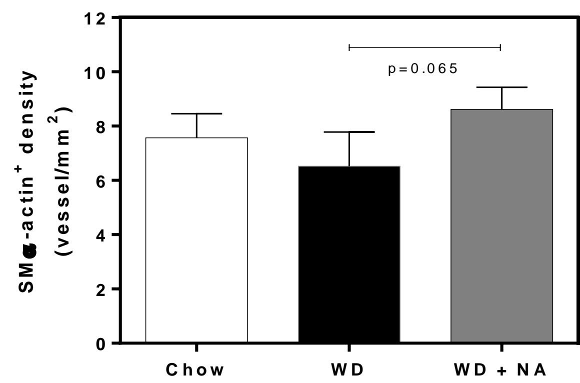

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Appendices

AUP Number: 2011-044
PI Name: Borradaile, Nica M
AUP Title: Vascular Regeneration After Increased Nad+ Availability In Diet Induced Metabolic Syndrome

Approval Date: 03/09/2012
Review Date: 03/09/2013
Expiry Date: 03/09/2016

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Vascular Regeneration After Increased Nad+ Availability In Diet Induced Metabolic Syndrome" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2011-044::1

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
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PUBLICATIONS

Hughes-Large JM, **Pang DKT**, Robson DL, Chan P, Toma J, Borradaile NM. Niacin receptor activation improves human microvascular endothelial cell angiogenic function during lipotoxicity. *Atherosclerosis* (2014). [Submitted]

PRESENTATIONS AND CONFERENCES

Hughes-Large J, **Pang D**, Robson D, Chan P, Sawyez C, Borradaile N. Activation of niacin receptor improves human microvascular endothelial cell angiogenic functions during lipotoxicity. Physiology and Pharmacology Research Day. Western University. November 7, 2013. [poster]

Pang D, Hughes-Large J, Chan P, Robson D, Sawyez C, Borradaile N. Niacin improves human microvascular endothelial cell angiogenic function under lipotoxic and hypoxic conditions. London Health Research Day. March 18, 2014. [poster]

Pang D, Hughes-Large J, Robson D, Chan P, Sawyez C, Borradaile, N. Niacin improves human microvascular endothelial cell angiogenic function under lipotoxic and hypoxic conditions. Arteriosclerosis, Thrombosis and Vascular Biology Scientific Sessions 2014. [poster]

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