

PALMITATE INDUCED ENDOTHELIAL DYSFUNCTION:
THE ROLE OF CALPAIN,
AMPK AND ENOS

A Thesis
Submitted to
the Temple University Graduate Board

In Partial Fulfillment
of the Requirements for the Degree
MASTER OF SCIENCE

by
Zhao Liu
August, 2014

Examining Committee Members:

Major Advisor, Rosario Scalia, MD. PhD, Physiology
Michael Autieri, PhD, Physiology
Laurie Kilpatrick, PhD, Physiology
Fabio Recchia, MD. PhD, Physiology

ABSTRACT

PALMITATE INDUCED ENDOTHELIAL DYSFUNCTION:
THE ROLE OF CALPAIN,
AMPK AND ENOS

By Zhao Liu

Master of Science

Temple University, August, 2014

Advisor: Rosario Scalia

Obesity is a serious health problem worldwide. Consumption of fat rich food is a common cause of obesity. Some of the food components (i.e. saturated free fatty acids (SFAs)) have been identified as inflammatory inducers (*Egger G et al., 2010*). After a meal, absorbed free fatty acids (FFAs) will be stored in the liver and adipose tissue. On the luminal surfaces of endothelium in adipose tissue microcirculation, lipoprotein lipase hydrolyses absorbed triglycerides into FFAs. Then, in order to be available for adipocyte storage, FFAs have to cross the capillary endothelium barrier, which connected by tight junctions (*Stremmel W et al., 2001*). Increased leukocyte infiltration is a featured sign of adipose tissue inflammation found in obesity. Endothelial adhesion molecules up-regulation contributes to leukocyte infiltration during inflammation. Some clinical data suggested an increase of leukocyte-endothelium interaction in healthy volunteers after ingestion of high-fat meals (*Shimabukuro M et al., 2007*). Other lab results also showed that neutrophil infiltration occurred at a very early stage with high-fat feeding in mice

(*Talukdar S et al., 2012*). However, the detailed mechanism of the above phenomena is still unknown.

This thesis provides exciting preliminary data which will guide the further study in this area. First of all, we successfully established a stable protocol that CD31 antibody conjugated microbeads were used to isolate primary microvascular endothelial cells from fresh mice lung tissue. After second sorting, CD31+ cells reach 83.3% by FACS analysis. Previous literatures showed that FFAs activate recruitment of inflammatory cells through up-regulation of endothelial adhesion molecules via reduced eNOS derived eNO production (*Rizzo NO et al., 2010; Davenpeck KL et al., 1994; Ahluwalia A et al., 2004*). In this thesis, it was found that SFAs palmitate exposure dose dependently reduced endothelial AMPK thr₁₇₂ and eNOS ser₁₁₇₇ phosphorylation by western blot. Moreover, our study demonstrated that endothelial calpain, a calcium dependent protease associated with endothelial dysfunction, was activated by palmitate, specifically its μ -calpain isoform. Altogether, these data suggested that a new role of calpain as a key mediator of palmitate induced endothelial dysfunction and indicated both AMPK and eNOS₁₁₇₇ phosphorylation contribute to this pathological process. Further investigations are still needed to explore connections among those molecules. This thesis may also lead to a novel way of clinical treatment for the obese related vascular diseases.

ACKNOWLEDGEMENTS

I am deeply indebted to those people who helped me to complete my study in Temple University. Without their help, it would be impossible to finish my thesis.

First of all, I would like to express my thankfulness to my advisor Dr. Rosario Scalia for his encouragement and enthusiasm. He is not only an excellent researcher, but also an outstanding advisor. It has been a great honor to be one of his students. From him I have learned the way to think creatively and critically in doing research projects. He has also trained and enhanced my trouble shooting and problem solving skills. I really appreciate all his time and effort during the past three years which helped me to gain progress every day. Additionally, I must express my gratitude for his patience in helping me to finish this thesis.

I sincerely thank my thesis dissertation committee members: Dr. Michael Autieri, Dr. Laurie Kilpatrick, Dr. Victor Rizzo and Dr. Fabio Recchia. Thanks for their advising, suggestions and tremendous support.

I really appreciate everybody in our lab and other laboratories that helped me with my research and life. Thanks for their friendship and assistance. Especially I owe deep thanks to these people: Inna Roma, Gavin Landesburg, Kyle Preston and Zienab Etwebi.

And last, I would like to thank my parents and my husband, who constantly supported and encouraged me. I would like to thank my friends and roommates for their help. They made me a wonderful life in Philadelphia.

TABLE OF CONTENTS

| | Page |
|---|------|
| ABSTRACT | i |
| ACKNOWLEDGEMENTS | iii |
| LIST OF FIGURES | vii |
| LIST OF TABLES | ix |
| | |
| CHAPTER | |
| 1. INTRODUCTION | 1 |
| Obesity and Nutrient Overload..... | 1 |
| Adipose Tissue Microcirculation and Meta-flammation | 1 |
| Adipose Tissue | 1 |
| Adipose Tissue Microcirculation | 2 |
| Adipose Tissue Inflammation..... | 3 |
| Fats and Free Fatty Acids Metabolism..... | 4 |
| Fats Digestion, Absorption and Storage..... | 4 |
| Free Fatty Acids | 6 |
| Endothelial Dysfunction..... | 8 |
| Normal Endothelium and Endothelial Dysfunction | 8 |

| | |
|---|----|
| Leukocyte-Endothelium Interaction | 9 |
| Signaling Molecules in Endothelial Dysfunction | 11 |
| Research Objectives and Hypothesis..... | 20 |
| 2. MATERIALS & METHODS | 22 |
| Animals..... | 22 |
| Cells and Culture | 22 |
| Endothelial Cell Lines | 22 |
| Primary Microvascular Endothelial Cell Isolation | 22 |
| Primary Culture of Freshly Isolated MECs | 24 |
| Materials..... | 25 |
| Free Fatty Acids Preparation | 25 |
| Biochemical Studies | 26 |
| Western Blot | 26 |
| Calpain Activity Assay..... | 27 |
| Flow Cytometry..... | 28 |
| Statistical Analysis..... | 28 |
| 3. RESULTS | 29 |
| Mice Microvascular Endothelial Cells Isolation..... | 29 |
| FACS Analysis of PECAM-1 Surface Expression in Cultured MECs..... | 31 |

| | |
|---|----|
| Palmitate Dose Dependently Reduces Phosphorylation Of eNOS ₁₁₇₇ , not eNOS ₆₃₃ | 32 |
| Palmitate Dose Dependently Reduces AMPK Phosphorylation..... | 35 |
| Calpain Activity Is Activated by Palmitate | 36 |
| μ-Calpain Instead of m-Calpain Isoform Is Activated by Palmitate | 39 |
| 4. DISCUSSION | 42 |
| Overview | 42 |
| Discussions..... | 43 |
| Conclusions..... | 49 |
| Future Directions..... | 49 |
| 5. REFERENCES CITED..... | 52 |

LIST OF FIGURES

| Figures | Page |
|--|------|
| 1. Figure.1-1 Schematic Illustration of How Adipose Tissue Iptake FFAs..... | 03 |
| 2. Figure.1-2 Hourly FFAs Concentration Measurement..... | 06 |
| 3. Figure.1-3 Endothelial Dysfunction..... | 09 |
| 4. Figure.1-4 Leukocyte Recruitment to Sites of Inflammation..... | 10 |
| 5. Figure.1-5 endothelial Nitric Oxide Synthase (eNOS)..... | 14 |
| 6. Figure.1-6 AMP-Activated Protein Kinase (AMPK) in endothelial cell..... | 17 |
| 7. Figure.1-7 Calpain Structure and Activation..... | 19 |
| 8. Figure.1-8 Working Hypothesis..... | 21 |
| 9. Figure.2-1 Microvascular Endothelial Cells Isolation from Fresh Animal Tissue..... | 24 |
| 10. Figure.3-1 Microscopic Morphology of Isolated Microvascular Endothelial Cells from Fresh Animal Tissue..... | 30 |
| 11. Figure.3-2 Flow Cytometry of PECAM-1(CD31) Staining for the Isolated Cells..... | 31 |
| 12. Figure.3-3 Palmitate Dose-dependently Reduces eNOS Ser ₁₁₇₇ Phosphorylation..... | 33 |
| 13. Figure.3-4 Palmitate Does Not Reduce eNOS Ser ₆₃₃ phosphorylation..... | 34 |
| 14. Figure.3-5 Palmitate Dose-dependently Reduces AMPK Phosphorylation..... | 36 |
| 15. Figure.3-6 Palmitate Acutely Activates Calpain..... | 38 |
| 16. Figure.3-7 μ -Calpain Isoform Is Activated by Palmitate..... | 40 |

17. Figure.3-8 m-Calpain Isoform Is Not Activated by Palmitate.....41

18. Figure.4-1 Schematic Illustration of Palmitate Induced Endothelial Adhesion
Molecule Up-Regulation.....43

LIST OF TABLES

| Tables | Page |
|---|------|
| Table.1-1 Summary of eNOS ser1177 and ser633 phosphorylation sites..... | 15 |

CHAPTER 1

INTRODUCTION

Obesity and Nutrient Overload

Obesity, defined as a BMI parameter ($\text{Mass (kg)} / \text{Height}^2 (\text{m}^2)$) greater than 30, has become a global health concern. Generally speaking, changes in diets and lifestyles that promote a positive energy balance are the main reasons for the increased number of people with obesity all over the world (*Vasanti S et al., 2013*). With the development of globalization, the western style diet which contains a large amount of calories, and a reduction in physical activity as a result of a mechanized lifestyle, are the major reasons for nutrient overload in the human body which is attributed to obesity (*Bauer KW et al., 2012*). Particularly, the typical American diet, which includes high intakes of red meat, sugary desserts, high-fat foods and refined grains, is the top cause of nutrient overload. Usually the typical American diet contains about a 35% of fat, which greatly exceeds the dietary guidelines for fat ($< 30\%$) (*Last, Allen R et al., 2006*). In addition, the largest portion of fat in the American diet is saturated fat.

Adipose Tissue Microcirculation and Meta-inflammation

Adipose Tissue

Along with nutrient overloading, adipose tissue is considered to play a very important role in promoting the development of obesity. Inside the human body, adipose tissue can be found almost in all anatomical areas. Those found under the skin or dermis are mainly referred to as subcutaneous adipose tissue (SAT) and those surrounding the internal organs are referred as visceral adipose tissue (VAT). VAT is composed of several adipose depots, including omental, mesenteric, perirenal and perigonadal fat

depots. It has been reported that visceral adipose tissue expansion is strongly associated with obesity, insulin resistant and cardiovascular diseases (*Haffner SM Et al., 2007*). Conversely, subcutaneous adipose tissue expansion does not exhibit similar passive results (*Kim JY Et al., 2007*).

Adipose Tissue Microcirculation

Once, adipose tissue was considered as an organ with a sparse vasculature. However, later studies have proven that adipose tissue is a heterogeneous tissue composed of both adipocytes and stromal vascular fraction (*Gersh I et al., 1945*). The microcirculation of adipose tissue includes arterioles, capillaries and venules. Arteriole is the site where blood flow was regulated and capillaries and venules are the main places for nutrient exchange by regulating vascular permeability. In the adipose tissue there are abundant lipoprotein lipases (LPL), a very important enzyme in the metabolism process of fats on the luminal surfaces of capillary endothelial cells. LPL hydrolyses triglycerides into Free Fatty Acids (FFAs) and glycerine, thereby making FFAs available for uptake through the surrounding tissue (Fig.1-1). In adipose tissue, LPL is synthesized and secreted by the adipocytes and is trans-located to the lumen of capillaries. It is then bound to the luminal surface of endothelial cells by interaction with cell-surface glycosaminoglycans (*Wong H et al., 1997*). In order to be hydrolyzed by LPL, GPI-anchored high-density lipoprotein-binding protein 1 (GPIHPB1) facilitates the binding of a chylomicron particle on the surface of the capillary microvascular endothelial cell, (*Ioka RX et al., 2003*). More interestingly, LPL activity is regulated tissue-specifically according to nutritional status. For example, post-prandial LPL activity in the adipose tissue is very high in order to store FFAs inside the adipocytes (*Pilz S et al., 2008;Bonnet*

M et al., 2000). Because of long time nutrient overloading, LPL activity is also elevated in obese people (*Boivin A et al., 2007*).

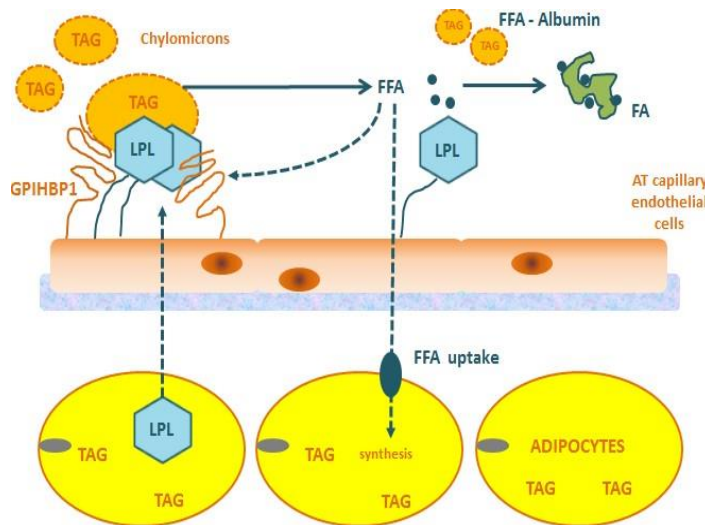


Figure.1-1: Schematic Illustration of How Adipose Tissue Intake FFAs. In the microcirculation of adipose tissue, LPL hydrolyzes lipoprotein-associated triglycerides into FFAs. These FFAs have to go through microcirculation endothelial cells in order to be stored inside the adipocytes (*Modified from Lafontan M. 2008*). FFA: Free Fatty Acids; TAG: Triglycerides; LPL: Lipoprotein Lipase; GPIHBP1: GPI-anchored high-density lipoprotein-binding protein 1.

Adipose Tissue Inflammation

With long time nutrient overloading, inflammation response could be found in the obese state. This metabolic product induced inflammation in response to excessive nutrients and energy is characterized as a chronic, low-grade type. It is named metaflammation (*Margaret F et al., 2011*). A range of food components have been identified as the nutritional meta-flammatory “inducers”, including saturated fatty acids (*Egger G et al., 2010*). Meta-flammation of obesity occurs mostly in expanding adipose tissue, especially intra-abdominal visceral ones (*Margaret F et al., 2011*). It is well known that

adipocyte has an endocrine function by secreting a lot of factors during inflammation. A large number of studies have found that by increasing circulation inflammatory factors, obesity, which relate to increased visceral adipose tissue mass, is also associated with many other systemic diseases, including cardiovascular disease (*Berg AH et al., 2005*).

Adipose tissue is the site where inflammation was first described and is most studied in obesity. An important feature of inflammation is the infiltration of inflamed tissues by circulating leukocytes such as neutrophils and macrophages. In fact, leukocyte infiltration of adipose tissue has been described in obese conditions of both mice and humans (*Furuhashi M et al., 2008*). Additionally, macrophage is the main leukocyte population that was increased in the adipose tissue of obese people (*Wellen KE et al., 2003*). In response to infectious and inflammatory signals, adipocytes have been shown to induce secretion of several mediators of inflammation and chemotactic signals which lead to immune cell recruitment (*Furuhashi M et al., 2008*). Under the effects of chemotactic factors, monocytes in the blood circulation will migrate into adipose tissue and become macrophage. This process is regulated by leukocyte-endothelium interaction which is mediated via adhesion molecules. A series of adhesion molecules were observed to be up-regulated inside adipose tissue and participate in leukocyte recruitment during obesity (*Roth Flach RJ et al., 2013; Bosanská L et al., 2010*).

Fats and Free Fatty Acids Metabolism

Fats Digestion, Absorption and Storage

Most fats in the human diet are in the form of triglycerides, which consist of three free fatty acids (FFAs) linked to glycerol. The digestion of dietary triglycerides starts in the stomach with the action of gastric lipase. Then, it continues in the duodenum with the

synergetic action of pancreatic lipase. In the small intestine, intestine lipases degrade triglycerides into FFAs and other breakdown products. Then they are taken up by the enterocytes and converted into triglycerides again. Those triglycerides are incorporated with cholesterol and apo-lipoproteins into chylomicrons. The Chylomicrons released from the cells move through the lymphatic system and bloodstream into tissues. In peripheral tissue, the capillary is covered only by one single layer of endothelial cells and a basement membrane. On the surface of endothelium, Lipoprotein Lipase (LPL) hydrolyzes triglycerides and releases FFAs from it. Those FFAs will enter into the tissue cells either for oxidation to provide ATP or be re-esterified for storage.

Some evidence in clinical research showed that in normal individuals, the influx of circulating plasma FFAs is immediately buffered after a meal (*Gerald M et al., 1988*) (Fig.1-2). This drop of FFAs plasma concentration is a potent evidence to indicate that FFAs storage into body tissues immediately after food intake. Liver and adipose tissue are the two main organs to store FFAs inside the body. In the liver, capillary endothelial cells are fenestral, allowing the passage of large molecules (ie. FFAs). So circulating FFAs could directly access the hepatocyte for storage. However, in adipose tissue, the capillary endothelial cells are connected by tight-junctions, a condition that prevents direct uptake of FFAs (*Stremmel W et al., 2001*). FFAs must cross the capillary endothelium in order to be available for adipocytes.

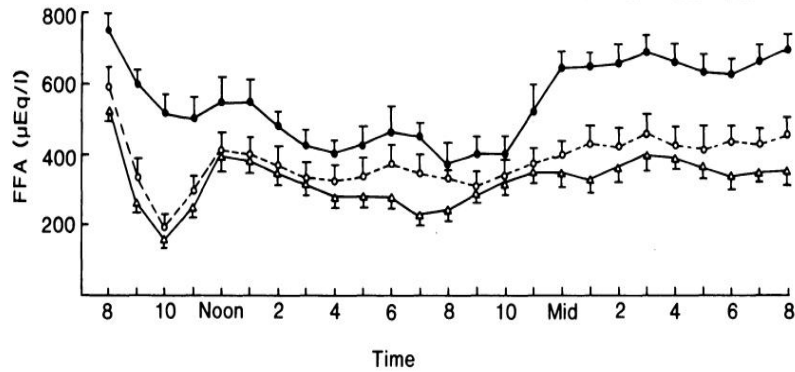


Figure.1-2: Hourly FFAs Concentration Measurement. Meals were eaten at 8am (20% of total calories), noon (40% of total calories), and 6pm (40% of total calories) in normal (Δ), mildly non-insulin-dependent diabetic (o), and severely non-insulin-dependent diabetic (\bullet) individuals. (Adapted from *Gerald M et al., 1988*)

Free Fatty Acids

Free fatty acids (FFAs), as mentioned above, are released principally through lipolysis of triglycerides. Because of large quantities of ATP production during metabolism, FFAs are important sources of energy for the human body. In the plasma, FFAs are bound with albumin to let them be transported through blood into the peripheral tissues. High concentrations of FFAs are associated with insulin resistance, fatty liver disease, hypertension, atherosclerosis and myocardial dysfunction (*Bays H et al., 2004*). Some clinical studies have shown that FFAs are elevated in patients with cardiovascular diseases (*Djoussé L et al., 2013*).

Basically, according to the number of their double bonds, FFAs are subdivided into Saturated Fatty Acids (SFAs), Monounsaturated Fatty Acids (MUFAs) and Polyunsaturated Fatty Acids (PUFAs). Or alternatively according to the number of their carbon atoms FFAs are categorized into short-chain (<8), medium-chain (8–12), long-

chain (13–22) or very-long-chain (>22) fatty acids (*Oram JF et al., 2004*). Regarding to the type of fat, cross-sectional research suggests that SFAs such as laurate, myristate, and palmitate, are pro-inflammatory, adversely affects vascular function whereas PUFAs such as arachidonate, eicosapentaenate (EPA), and docosahexaenate (DHA) are anti-inflammatory, beneficial. For example, EPA and DHA can reduce blood pressure, improve arterial compliance in type 2 diabetics and increase endothelium-dependent vasodilation (*Hall WL et al., 2009*).

Within the circulation, FFAs exist as a FFA-albumin complex. Thus, dissociation from albumin represents the initial step of FFAs cellular uptake process. This process involves membrane proteins with high affinity for fatty acids. Both passive diffusion and protein-facilitated transport contribute to the transmembrane movement of FFAs (*Hamilton JA et al., 1999; Frohnert BI et al., 2000; Hajri T. et al., 2002; Doege H et al., 2006; Kampf JP et al., 2007*). So far, diverse proteins have been identified in this process, including plasma membrane associated fatty acid binding protein (FABP); fatty acid translocase (FAT/CD36); fatty acid transport proteins (FATP1-6) and caveolin. They are known to be regulated by hormones such as insulin or by the energy requirements of the cells (*Stremmel W et al., 2001*). FFAs that arrive in the cytosol are transported into the mitochondria, then converted to acyl-CoA, which finally undergoes oxidation. Apart from their significance in the production of energy, FFAs also play an important role as substrates in many other cellular processes such as membrane biosynthesis, protein modification, transcription regulation and intracellular signal transduction.

Although the underlining mechanism is still unknown, there were some evidences of excess free fatty acids induced endothelial dysfunction in conditions of nutrient

overload. Some clinical data shows that increased leukocyte-endothelium interaction in healthy volunteers after uptake of high-fat meals, which is accompanied by the increased FFAs concentration (*Shimabukuro M et al., 2007*). Other labs also report that neutrophil infiltration occurs very early with the administration of high-fat feeding in the mice study (*Talukdar S et al., 2012*).

Endothelial Dysfunction

Normal Endothelium and Endothelial Dysfunction

The endothelium is a single layer of endothelial cells covering the vascular lumen. This cell layer was thought to be metabolically active and it is quite important in the maintenance of vascular homeostasis under physiological conditions (*P. Vallance et al., 2001; P.O. Bonetti et al., 2003*). In detail, the multiple functions of vascular endothelium include regulation of vessel integrity and permeability, vascular growth and remodeling, as well as angiogenesis. Moreover, the endothelium plays a pivotal role in regulating vascular tone, controlling tissue blood flow and inflammatory responses (*M. Félétou et al., 2006; S. Moncada et al., 2006; M. Félétou et al., 2011*).

When the normal endothelium function becomes inadequate, it comes to endothelial dysfunction. The occurrence of endothelial dysfunction is usually in response to a pathologic stimulation. The impaired NO bioavailability is a hallmark of endothelial dysfunction. Additionally, endothelial dysfunction is characterized by one or more of the following features: increased expression of adhesion molecules, reduced endothelium-mediated vaso-relaxation, a tendency to thrombosis, overproduction of growth factors, excessive generation of ROS, increased oxidative stress, and enhanced permeability of the endothelial cells (*S. Taddei et al., 2003; M.H. Laughlin et al., 2008; W.T. Cade et al.,*

2008; F. Addabbo et al., 2009; A. Hirose et al., 2010) (Fig.1-3). Endothelial dysfunction, and especially the up-regulated endothelium adhesion molecules, is also considered to be one of the factors that lead to the adipose tissue inflammation in obesity.

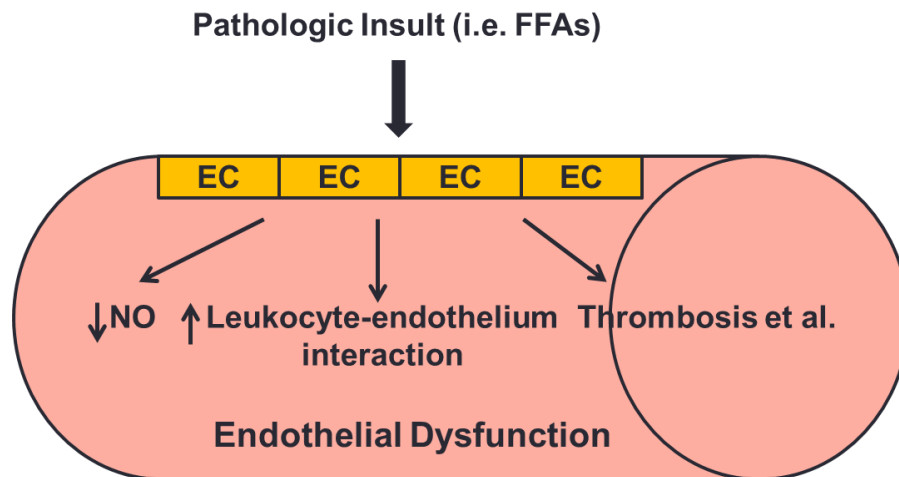


Figure.1-3: Endothelial Dysfunction. Endothelial dysfunction is characterized by one or more of those features including increased leukocyte-endothelium interaction, impaired eNO availability and thrombosis et al. EC: Endothelial Cell; FFA: Free Fatty Acids; NO: Nitric Oxide.

Leukocyte-Endothelium Interaction

One of the most important characteristics of endothelial dysfunction is the increased interaction between circulating leukocytes and endothelial cells. Leukocytes are divided into polymorphonuclear cells including neutrophils, basophils, eosinophils and mononuclear cells including lymphocytes and monocytes. The most abundant cell component of leukocyte is neutrophils. During inflammation, the process of leukocyte recruitment into inflammatory sites is mediated by its interaction with endothelial cells.

Leukocyte-endothelium interactions occur in three steps, and each step is mediated by a specific type of cell adhesion molecules (eCAMs) expressed on the surface of endothelial cell (*Krieglstein CF et al., 2001*). First of all, leukocyte rolling is regulated by the selectin family of adhesion molecules (i.e. P-selectin, E-selectin). Then, leukocyte adherence is mediated by the immunoglobulin family of adhesion molecules (i.e. ICAM-1 and VCAM-1). Finally, leukocyte extravasation into the inflammatory site is regulated by ICAM-1 and PECAM-1(Fig.1-4).

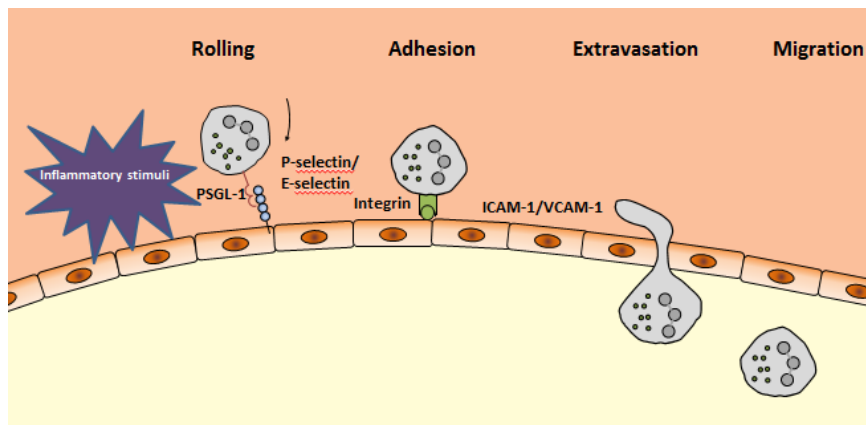


Figure.1-4: Leukocyte Recruitment into Sites of Inflammation. The circulating leukocyte must recognize signs of inflammation and migrate into areas where elimination of infection is needed. Selectin-mediated rolling along endothelium followed by integrin-mediated adhesion, subsequently, the leukocyte traverses through the endothelium and arrives at the site of inflammation. ICAM-1: intercellular adhesion molecule-1; VCAM-1: vascular cell adhesion molecule-1. PSGL-1: P-selectin glycoprotein ligand-1.

Signaling Molecules in Endothelial Dysfunction

Endothelial Cell Adhesion Molecules

Among all the families of cell adhesion molecules that participate in leukocyte trafficking, selectins and their glycoprotein ligands are the first two adhesion molecules that become up-regulated during inflammation. Selectins, including E-selectin and P-selectin, combine with their glycoprotein ligands for the tethering, rolling, and weak adhesion of leukocytes. Subsequently, integrins react with the immunoglobulin superfamily of cell adhesion molecules including ICAM-1(Intercellular Adhesion Molecule-1) and VCAM-1(Vascular Cell Adhesion Molecule-1), for firm adhesion and downstream signal transduction, which eventually triggers leukocyte extravasation. The leukocytes are then guided by chemo-attractants to migrate into their destinations where the inflammation occurred. It is generally believed that the selectin family of cell adhesion molecules are quickly up-regulated after inflammatory stimuli, which means they mediate the initial step of leukocyte-endothelium interactions during the recruitment of leukocytes (*Geng J. G. 2003; Vestweber D. et al., 1999*). While up-regulation of ICAM-1, VCAM-1 is much slower, their de novo synthesis takes several hours to happen, which means they mediate the later steps of leukocyte-endothelium interaction.

There are several studies that shed a light on the effects of free fatty acids, particularly inflammatory inducer saturated free fatty acids on the expression of endothelial adhesion molecules. First of all, free fatty acids up-regulate endothelial ICAM-1 and VCAM-1 expression through NO reduction. Chongxiu Sun et al. reported that triglyceride-rich lipoproteins (TGRL) modulate VCAM-1 production in the human aortic endothelial cells (HAEC) as a function of its FFA constituents (*Sun C et al., 2012*). Research group of Chandan Shrestha et al. demonstrate that palmitate activates

endothelial ICAM-1 and VCAM-1 expression through inducing the release of histone H3 (*Shrestha C et al., 2013*). A clinical study led by Manoj Mathew et al. displayed that elevated plasma FFA increased serum ICAM-1 and VCAM-1, the plasma markers of endothelial activation (*Mathew M et al., 2010*). NF-kB is a very important transcription factor that regulates ICAM-1 and VCAM-1 surface expression. Francis Kim's lab published that NO is the major mediator of palmitate induced endothelial cellular inflammation characterized by NF-kB activation and ICAM-1, VCAM-1 up-regulation (*Rizzo NO et al., 2010*). Furthermore, some research groups found that Inflammation quickly up-regulates P-selectin at the endothelial cell surface to initiate tethering of circulating leukocytes (*Ichimura H et al., 2003; Takano M et al., 2002*). In endothelial cells, endothelial nitric oxide (eNO) is a major inhibitor of Weibel-Palade body exocytosis, which further influences the surface expression of P-selectin (*Matsushita K et al., 2003*). Therefore, loss of physiologic levels of eNO leads to up-regulation of P-selectin (*Davenpeck KL et al., 1994; Ahluwalia A et al., 2004*).

Endothelial Nitric Oxide Synthase

Nitric oxide synthases (NOSs) are a family of enzymes catalyzing the production of nitric oxide (NO) from L-arginine. There are three known isoforms. They are neuronal nitric oxide synthase (nNOS), inducible nitric oxide (iNOS) and endothelial nitric oxide (eNOS) (*Stuehr DJ. et al., 1999*). eNOS is composed of two globular protein (reductase and oxygenase) interacted by a flexible protein strand, which have a specific calcium-dependent calmodulin (CaM) binding site (Fig.1-5). The reductase domain generates the electrons required for nitric oxide (NO) synthesis, electrons was transported through three cofactors of eNOS: NADPH, FMN and FAD. The electrons are then transferred across

protein strands in the middle to the oxygenase domain. The oxygenase domain consists of the catalytic center responsible for NO production as well as L-arginine and two other cofactors heme and BH₄ binding sites. Binding of CaM to its specific binding site increases the rate of electron transfer from the reductase domain to the catalytic center of eNOS, which is an initiator of eNOS activation (*Davignon J. et al., 2004; Mount PF et al., 2007*). NO produced by eNOS is a very important factor in maintaining the physiological function of vasculature. Human studies showed that NO production is impaired in the presence of high circulating FFAs concentration. It appears that the phosphorylation of Ser and Thr of eNOS by protein kinases is important for its enzymatic activity in vascular endothelial cells (ECs) (*Sessa WC et al., 2004*). Until now, 5 Ser/Thr phosphorylation sites in eNOS have been identified. They are Ser114, Thr495, Ser615, Ser633, and Ser1177 (*Mount PF et al., 2007*). Among all, Ser633 and Ser1177 are functioning as stimulatory phosphorylation sites (*Fulton D et al., 1999; Bauer PM et al., 2003; Michell BJ et al., 2001*) (Table.1-1). The phosphorylation of Ser1177 has been suggested to be critical to the eNOS activation responding to several stimuli including HMG-CoA reductase inhibitors, adiponectin and shear stress by activating multiple protein kinases such as AMP-activated protein kinase (*Dimmeler S et al., 1999; Chen H et al., 2003; Harris MB et al., 2004*).

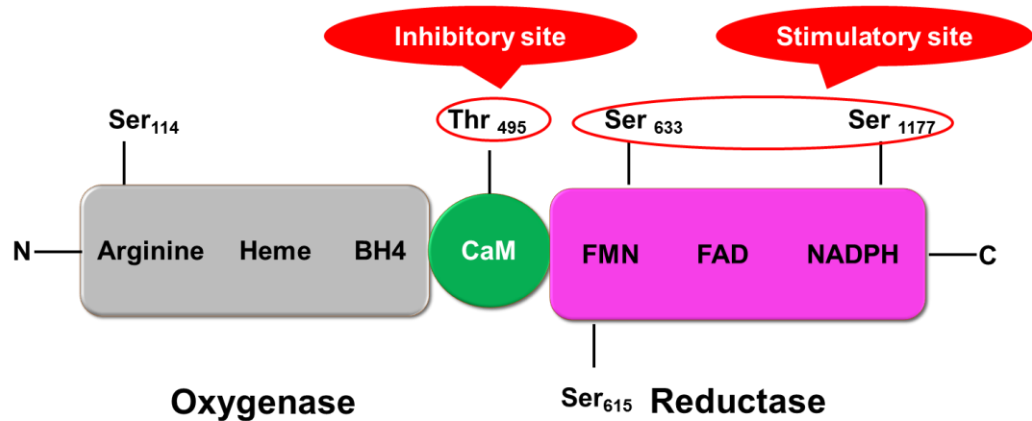


Figure.1-5: endothelial Nitric Oxide Synthase (eNOS). eNOS is composed of 2 globular protein modules (reductase and oxygenase) connected by a flexible protein strand with CaM binding site. The reductase domain generates the electrons required for nitric oxide (NO) synthesis. The oxygenase domain consists of the catalytic center responsible for NO production. There are 5 Ser/Thr phosphorylation sites in eNOS, Ser₆₃₃ and Ser₁₁₇₇ are stimulatory and Thr₄₉₅ is inhibitory. NADPH: nicotinamide adenine dinucleotide phosphate; BH₄: tetrahydrobiopterin; CaM: calmodulin; FAD: flavin adenine dinucleotide; FMN: Flavin mononucleotide. (Modified from *Mount PF et al., 2007*)

| Summary of eNOS Ser₁₁₇₇ and Ser₆₃₃ phosphorylation sites | | | |
|---|--|---------------|----------------------|
| Stimulation | Signalling pathway | eNOS site Ser | References |
| Vigorous exercise and ischemic stress | AMPK-eNOS | 1177 | Chen ZP. Et al |
| VEGF/IGF-1 | VEGF/IGF-1—IP3K—Akt--eNOS | 1177 | Michell BJ. Et al. |
| Shear stress | PI3K-Akt-eNOS | 1177 | Dimmeler S. et al. |
| Insulin | Akt-eNOS | 1177 | Montagnani M. et al. |
| Bradykinin | PI3K/Akt-eNOS | 1177 | Harris MB. Et al. |
| Bradykinin | PKA-eNOS | 633 | Michell BJ et al. |
| Shear stress, VEGF, 8-bromocAMP | PKA-PI3K w/o-eNOS | 633 | Boo YC et al. |
| AICAR | AMPK-eNOS | 1177 | Morrow VA et al. |
| Statin | PI3K/Akt-eNOS PKA | 1177 633 | Harris MB et al. |
| Palmitate | PP2A-AMPK-ACC (other pathway may also exist)-eNOS | 1177 | Wu Y et al. |
| VEGF,S1P | AMPK-Rac1-PI3K-Akt-eNOS | 1177 | Levine YC et al. |
| Shear stress, atorvastatin, adiponectin | AMPK | 633 | Chen Z et al. |
| Bradykinin | CaMKK-AMPK-eNOS | 1177 | Mount PF et al. |

Table.1-1: Summary of eNOS Ser₁₁₇₇ and Ser₆₃₃ phosphorylation sites.

AMP-Activated Protein Kinase

The AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine protein kinase consisting of a catalytic subunit (α) and 2 regulatory subunits (β and γ). It is activated by increased intracellular concentrations of AMP. Two major AMPK kinases have been identified until now. They are the tumor suppressor gene product LKB1 and the Ca^{2+} /calmodulin-dependent protein kinase kinase (CaMKK) (*Sanders MJ et al., 2007*). There are also some physiological and pharmacological stimuli including shear stress, adiponectin, HMG-CoA reductase inhibitors could activate AMPK (*Fulton D et al., 1999; Chen ZP et al., 1999; Zhang Y et al., 2006; Michell BJ et al., 2001; Boo YC et al., 2002*). Hence, AMPK is generally referred to as a “metabolic master switch”. Activation of AMPK markedly influences the activation of other signaling cascades. AMPK phosphorylates and inhibits ACC, increasing FFAs oxidation and reducing fatty acids synthesis (*Kudo N et al., 1995; Dagher Z et al., 1999*). There are numerous reports of attenuated NF- κ B activation following AMPK activation in different cell types, including endothelial cells (*Cacicedo JM et al., 2004; Okayasu T et al., 2008; Suzuki K et al., 2008; Hattori Y et al., 2008*). Some studies have shown that AMPK regulates eNOS activity at the posttranslational level. Additionally, AMPK could activate eNOS by phosphorylating either 1177site or 633site, inducing the eNOS-derived NO bioavailability. Interestingly, high-fat diet feeding reduces AMPK activity in multiple tissues of laboratory animals (*Lindholm CR et al., 2012*). Furthermore, Wu Y found that SFAs acutely reduces AMPK activity in endothelial cells (*Wu Y et al., 2007*). Conversely, stimulation of AMPK activity attenuates activation of pro-inflammatory pathway and increases NO release in human endothelial cells exposed to SFAs (*Mugabo Y et al., 2011*). Thus, it is reasonable to hypothesize that excess SFAs formation following ingestion of high-fat meals may

cause loss of basal eNO levels and subsequently up-regulation of adhesion molecules via down-regulation of AMPK activity.

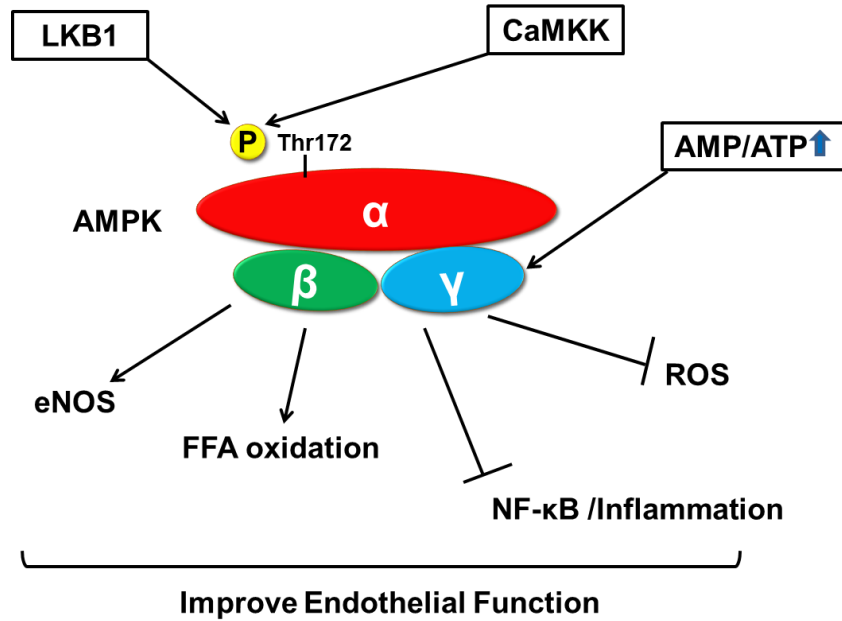


Figure.1-6: AMP-Activated Protein Kinase (AMPK) in Endothelial Cell. LKB1: liver kinase B-1; CaMKK: Ca^{2+} /calmodulin-dependent protein kinase kinase; AMP: Adenosine monophosphate; ATP: Adenosine triphosphate; ROS: Reactive Oxygen Species; FFA: Free Fatty Acids; NF- κ B: Nuclear Factor kappa-light-chain-enhancer of activated B cells.

Calpain

Calpain is a calcium-dependent cysteine protease that can proteolyze many cellular proteins. Since calpain cleaves a huge amount of proteins, including cytoskeletal molecules and signal transduction molecules, the calpain system is participated in many cell processes such as cell motility and cell cycle (*Goll DE et al., 2003*). Calpain family includes at least 15 isoforms. The ubiquitous and well known m and μ - calpain isoform

are named according to calcium concentration requirements for its activation: μ for micromole, m for millimole. In general, calpain is a heterodimer composed of an 80 kDa catalytic subunit and a 28 kDa regulatory subunit encoded by different genes. The larger catalytic subunit is comprised of an amino-terminal end that is autolyzed upon protease activation (domain I); a calcium-dependent catalytic site of cysteine, histidine, and asparagine residues (domain II); a phospholipid binding site for membrane targeting (domain III); and calcium binding site (domain IV). The smaller regulatory subunit also contains calcium binding sites and autolyzes the amino-terminus when activated. Upon activation, calpain will migrate to the cell membrane where it proteolyzes other downstream molecules (Fig.1-5).

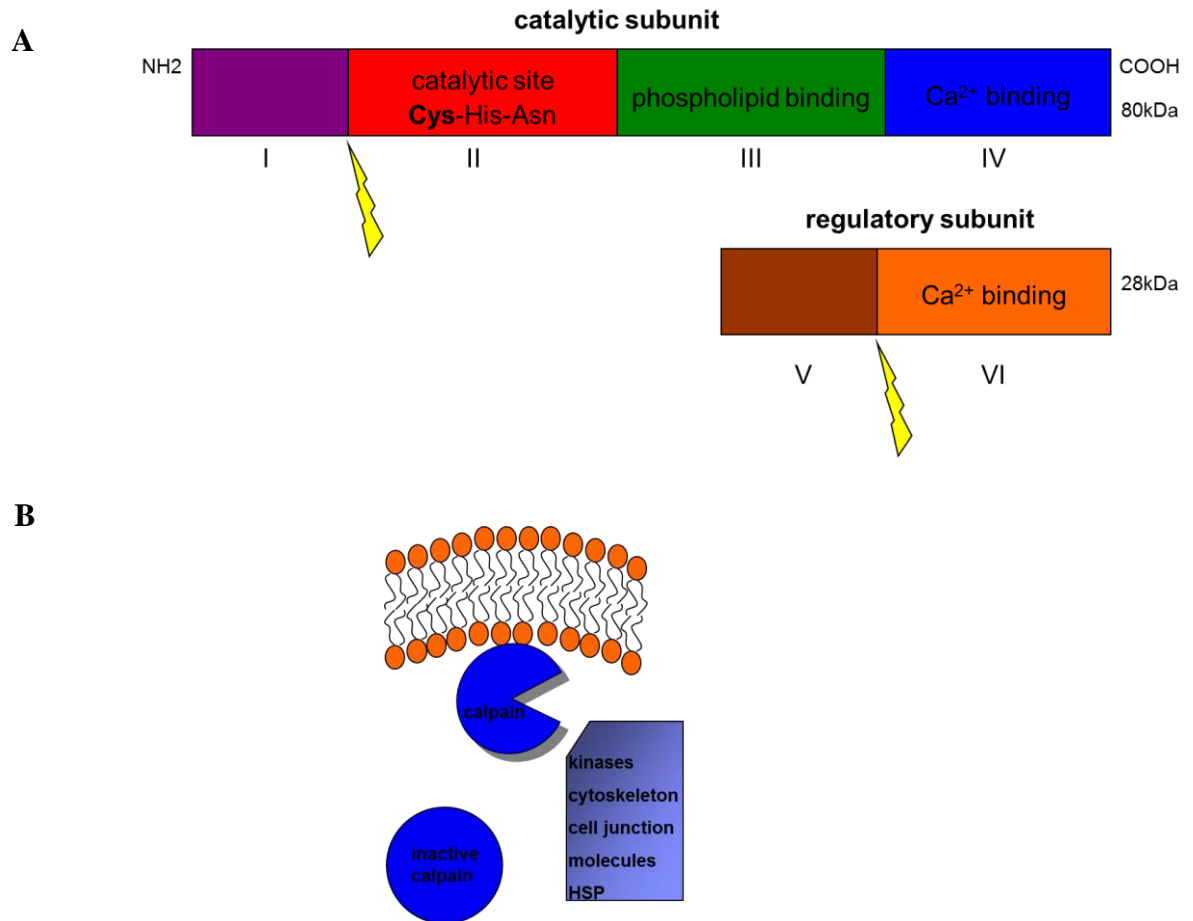


Figure 1-7: Calpain Structure and Activation. **A.** The protease calpain is a heterodimer. The amino-terminal ends of domain I and domain V are autolyzed upon calpain activation. Yellow jagged lines depict autolysis. **B.** Upon activation, calpain will migrate to the cell membrane and proteolyzes other molecules.

It has been reported that abnormal calpain activity is associated with cardiovascular system dysfunction, thereby indicating a strong relevance for studying protease calpain in this area. Specifically, calpain has also been implicated in endothelial dysfunction, which is commonly defined as the loss of endothelial nitric oxide (eNO) (Monica Averna *et al.*, 2008). More importantly, studies showed that conditioned medium from palmitate-treated astrocytes elevated the calcium level in primary cortical

neurons and increased calpain activity (*Li Qi Liu et al., 2013*), which indicates calpain could be activated by SFAs in the neuron. Furthermore, there is some data that prove calpain inhibition prevented leukocyte recruitment in the heart after ischemia/reperfusion injury via a reduction in P-selectin expression on microvessels (*Ikeda Y et al., 2005*). Thus, calpain inhibitors could be a potential target for future clinical treatment and drug development.

Research Objective and Hypothesis

The overall goal of this study is to establish a stable method to isolate microvascular endothelial cells from fresh animal tissue and explore the effects of SFAs palmitate in the expression of several signaling molecules that induce endothelial dysfunction. Given that palmitate exposure activates recruitment of inflammatory cells through up-regulation of endothelial adhesion molecules via reduced NO production. Our overall hypothesis is that AMPK, eNOS and calpain are downstream signaling effectors of palmitate induced endothelial dysfunction. In particular, Palmitate activates calpain, while down-regulates AMPK and eNOS expressions (Fig.1-8).

In order to test hypothesis made in this thesis, the following experiment designs will be addressed:

1. Isolate Microvascular Endothelial Cells from mice lung tissue by CD31 antibody-conjugated microbeads.
2. Measure the purity of CD31+cells by FACS analysis.
3. *In vitro*, studying the effects of palmitate on AMPK phosphorylation

4. *In vitro*, exploring the effects of palmitate on different eNOS phosphorylation sites, eNOS ser1177 and ser633 separately.
5. *In vitro*, studying the effect of acute palmitate exposure on endothelial calpain activity. Prove if this effect could be inhibited by calpain specific inhibitors
6. *In vitro*, detecting which specific calpain isoform plays a role in it, μ -calpain or m-calpain.

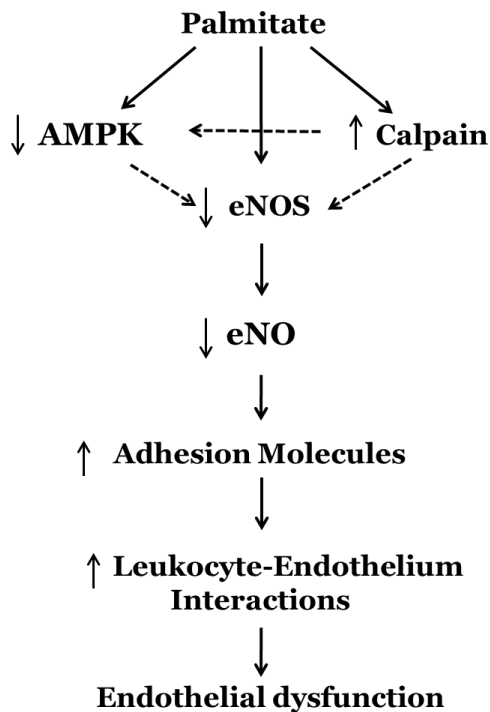


Figure.1-8: Working Hypothesis. Palmitate induces endothelial dysfunction through up-regulation of Adhesion Molecules. Three potential signaling molecules AMPK, eNOS, calpain have been found in palmitate pathway. Their potential relationships were also shown. (Dash line arrow is hypothesis not proved by this thesis). AMPK: AMP-activated protein kinase; eNOS: endothelial Nitric oxide synthases; eNO: endothelial nitric oxide.

CHAPTER 2

MATERIAL & METHODS

Animals

All animal experiments were performed in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the Temple University Institutional Animal Care and Use Committee guidelines. In this project, we used the wild type C57BL/6 mice. All animal procedures followed Temple University IACUC-approved protocols and conformed to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (*NIH, 8th ed., 2011*).

Cells and Culture

Endothelial Cell Lines

Bovine Aortic Endothelial Cells (BAECs) and Rat Heart Microvascular Endothelial Cells (RHMECs) were obtained as cryopreserved secondary culture from Vec Technologies, Inc. (*RENSSELAER, NY*). Then sub-cultured in MCDB131 medium (*Sigma-Aldrich, St. Louis, MO*) with 15% Fetal Bovine Serum (FBS). Cells were used from passages 3-5. Endothelial Cells were incubated in MCDB 131 medium without FBS for 24 hours starvation, and then exposed to stimulation.

Primary Microvascular Endothelial Cell Isolation

Our lab has successfully isolated Microvascular Endothelial Cells (MECs) from lung tissue, as previously described by our laboratory (*Stalker TJ et al., 2005*). Three to four 3-week old C57BL/ 6 wild type mice were used in the procedure every time. The mice were euthanized under anesthesia and lung tissues were excised immediately after

chest exposure. Avoid large vessels in the hilus, cut to get the peripheral lung tissue. These excised tissue samples were pooled in ice-cold Endothelial Base Medium (*EBM-2*, Lonza, Basel, Switzerland), remove large vessels, then minced samples and digested with 9 ml 0.1% Collagenase type I (*Gibco*, Grand Island, NY) and 1 ml Dispase (*STEMCELL Technologies Inc.* Vancouver, Canada) solution for 1 hour at 37 °C using a water bath shaker. The tubes were agitated for a few seconds at 5 min intervals during this incubation. After digestion, undigested tissue was removed from the cell suspension by filtration through a 100 µm cell strainer. The filtered cell suspension was then centrifuged for 5 min at 400×g. After removal of the floating cell layer and supernatant, the cell pellet was re-suspended in 5 ml of EBM2, and then filtered through a 40 µm cell strainer. Centrifuge the tube again to wash the cells, discard supernatant, re-suspend in 1 ml of EBM2. Cell counts and viability assessment were performed. Add 90 µL of beads wash buffer and 10 µL of MACS CD31 MicroBeads (*MicroBeads conjugated to monoclonal anti-mouse CD31 antibodies*, Miltenyi Biotech, San Diego, CA) per 10⁷ total cells to the cell pellet, according to the manufacturer's recommended protocol. The cells were incubated with Microbeads at a 1:10 dilution for 15-20 min at 4 °C. During this incubation, the LD columns were loaded onto MultiMACS separation units (*Miltenyi Biotech*, San Diego, CA) and each column was washed with 1 ml of buffer. After the beads had reacted, the cells were washed with 1 ml of MACS buffer and the cell pellet was re-suspended in 500 µl of buffer. The resulting suspension was then applied to the washed column to start the first sorting. Since the Microbead-coated CD31+ cells were retained on the LD columns, the flow through was deleted (*van Beijnum JR1 et al.*, 2008).

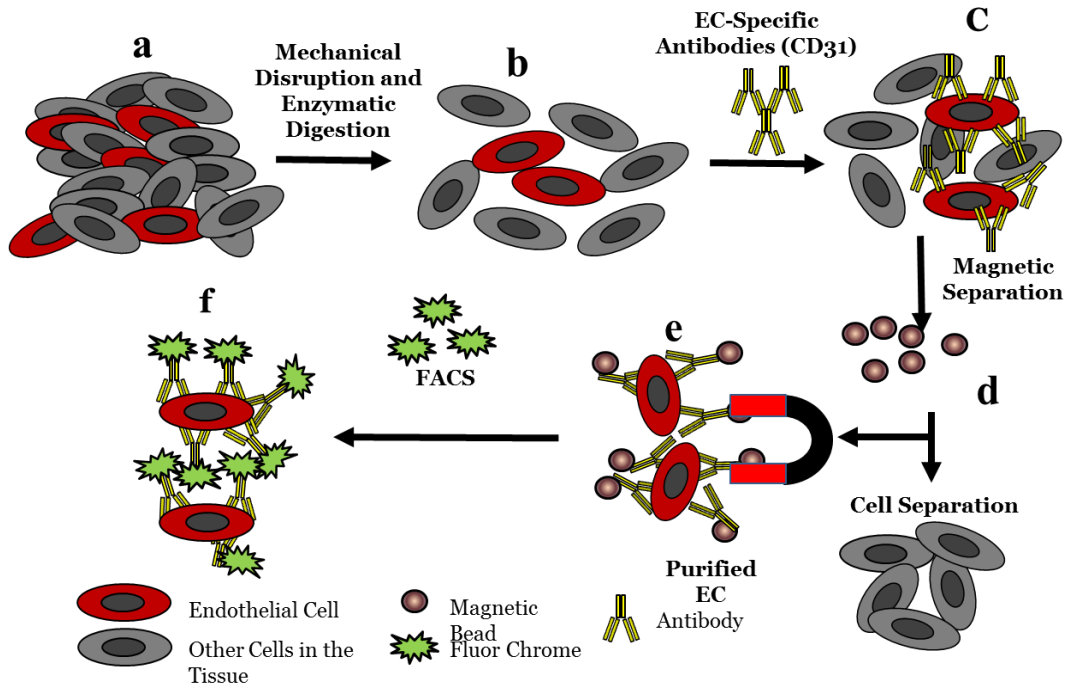


Figure.2-1: Microvascular Endothelial Cells Isolation from Fresh Animal Tissue

(Modified from van Beijnum JR.2008)

Primary Culture of Freshly Isolated MECs

Immediately after separation, centrifuge the collected cells at 400xg 5 min, discard supernatant. Re-suspend the cell pellet in 2 ml MCDB 131 complete medium with 5% FBS. Pour the mix on to one well of a six well plate (pre-coated with 0.1 % gelatin). Medium were changed every other day. After confluent, detach the cells using 1mM EDTA, some of the cells were used for Flow cytometry of CD31+ cells to confirm the purity of isolation (see *flow cytometry*). Other cells were continuously cultured, used for the second sorting.

Materials

Free Fatty Acids Preparation

FFAs were prepared by conjugation with albumin, as described previously (Svedberg J *et al.*, 1990). Palmitic acid (*Sigma*) was first dissolved in NaOH at 100 mM and conjugated with Bovine Serum Albumin (*BSA, sigma*) at a molar ratio of 3:1 (Palmitate/albumin). Control solution containing NaOH and BSA was similarly prepared. All FFAs were freshly prepared to prevent oxidation. All reagents used were tested for endotoxin level using the Limulus amoebocyte lysate (LAL) test kit (*Sigma*).

Pharmacological Intervention

Enzyme activity was blocked *in vitro* with pharmacological inhibitors. Pharmacological inhibition of enzyme activities introduces the possibility of non-specific results due to the chemical properties of each inhibitor. Accordingly, multiple pharmacological inhibitors for each target were used to confirm the specificity of the results.

Z-Leu-Leu-CHO (ZLLal) is a dipeptide that binds the catalytic site of calpain and is selective for calpain over other proteosomal enzymes (*Tsubuki S et al.*, 1996). The concentration of ZLLal used in these studies was previously demonstrated to be highly selective for calpain over caspase (*Tsubuki S et al.*, 1996). Calpain inhibition was achieved *in vitro* with 100uM ZLLal. Stock concentrations of ZLLal were prepared in dimethyl sulfoxide (DMSO). ZLLal was diluted in the supportive cell culture medium to the final concentration for *in vitro* studies.

PD150606 is a benzene structure that interferes with the calcium-binding site of calpain, thus preventing calcium activation of calpain (*Stalker TJ et al.*, 2003).

Therefore, PD150606 inhibits calpain with a different mechanism of action and chemical structure than ZLLal. Calpain inhibition was achieved *in vitro* with 100uM ZLLal. Stock concentrations of ZLLal were prepared in dimethyl sulfoxide (DMSO). ZLLal was diluted in the supportive cell culture medium to the final concentration for *in vitro* studies.

Biochemical Studies

Western Blot

Briefly, cells were rinsed with PBS and Lysis buffer (*M-PER Mammalian Protein Extraction Reagent, Thermo Scientific*) with 1:1000 Proteinase Inhibitor cocktail (*sigma*) were added to cover the cell, then scraped all the cell extracts. Subsequently, the samples were ultra-sonicated at 0.03 Watts for 5-10 seconds, and centrifuged at 14,000 rpm for 15 minutes. The supernatant was collected and assayed for protein content according to the BCA method (*Thermo Scientific, Waltham, MA*). Equal amounts of protein (15 µg) were prepared in loading buffer containing. The samples were then boiled at 100°C for 3-5 minutes for protein denaturation. SDS-PAGE was performed by running samples through a polyacrylamide gel (*10% ready gel, Bio-Rad*) at a constant current. Protein was transferred to a poly-vinylidene fluoride (PVDF) membrane in transfer buffer at a constant voltage at 4°C. The membranes were then blotted for protein expression. Non-specific binding of antibodies was blocked by incubating the membranes in Tris-buffered saline + 0.1% Tween (0.1% TBS-T) containing 5% milk for 1 hour at room temperature. After washing in 0.1% TBS-T, the membrane was incubated with the primary antibody. Membranes were washed again in 0.1% TBS-T and then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody. A chemiluminescent substrate

(*Thermo Fisher Scientific Inc., Rockford, IL*) was then added to the membrane. The signal was visualized using a Fuji LAS-4000 imaging machine (*Fujifilm Holdings, Tokyo, Japan*). Images were analyzed by densitometry using Image J software (*National Institutes of Health, Bethesda, MD*).

Western Blot Analysis of μ -Calpain and m-Calpain

Activity of the μ -calpain and m-calpain isoforms expressed in endothelial cells was studied by western blot analysis, using monoclonal antibodies against either the activation-cleaved N-terminus domain (*RP1 calpain-1, RP1 calpain-2*) or the stable Domain IV (*RP3 calpain-1; RP3 calpain-2; Triple Point Biologics, Portland OR*) of the large subunit of μ -calpain and m-calpain respectively (*Wang KK et al., 1996*). Calpain autolyzes the amino-terminal end of domain I upon activation. The resulting loss of amino-terminal domain antibody detection can be used as a measure of μ -calpain and m-calpain activation (*Shah V et al., 1999*). Antibodies against the stable domain IV of μ -calpain were used to measure total μ -calpain and m-calpain expression levels.

Calpain Activity Assay

A modified protocol was used to measure calpain activity in Bovine Aortic Endothelial Cells. Cells were cultured in the 24 well plates until confluent, then were incubated with 60 μ M Succ-LLVY-AMC in PBS containing Ca^{2+} for 30 minutes at 37°C. Fluorescence of each well (excitation λ 360 nm; emission λ 460 nm) was measured with a Bio-Tek FLx800 microplate fluorescence reader (*Bio-Tek, Winooski, VT*) for the basal reading. Cells were then treated with Palmitate-BSA or BSA for different time. After that, fluorescence was read again. Calpain activity was calculated as the delta change in fluorescence intensity from the fluorescence reading after main treatment (I_0) to the

fluorescence reading after substrate incubation(I_f) according to the equation $\Delta I = I_f - I_0$. Specificity of the Succ-LLVY-AMC for calpain was confirmed by pharmacological inhibition.

Flow Cytometry

CD 31 expression level in 2×10^6 isolated MECs was performed by FACS analysis using an anti-rat FITC-conjugated antibody for CD31 (*BD Biosciences, USA*) or control isotype IgG according to standard procedure also reported by the our team (*Scalia R et al., 1997*). Briefly, antibodies were added for 40 min at 4°C followed by extensive washing with PBS, and then analyzed by FACS. Data was analyzed by software FlowJo (*Three Star Inc. Ashland, OR*).

Statistical Analysis

Statistical analyses were performed using analysis software (*Graphpad 5.0; Prism*). Results are expressed as mean \pm SEM. Differences between groups were evaluated using one-way ANOVA. Individual mean differences were evaluated using the Newman-Keuls post-test and confirmed by paired t-tests where appropriate. Differences were considered significant at a level of $p < 0.05$.

CHAPTER 3

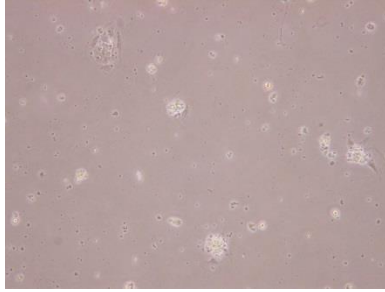
RESULTS

Mice Microvascular Endothelial Cells Isolation

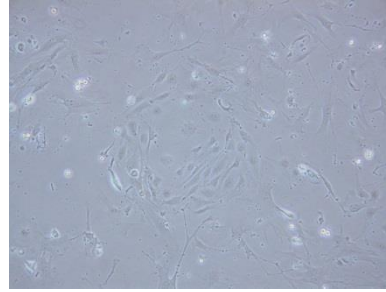
It is well known that leukocyte infiltration into organ tissue through interaction with endothelium of post-capillary venules happens in the microcirculation. Moreover, the largest single concentration of endothelial cells in the body (>25%) is present in the pulmonary microvasculature (*Hewett PW et al., 1993*). So we decided to isolate microcirculation endothelial cells from mice lung tissues. Isolation procedure was performed as our protocol mentioned in chapter 2. After first sorting by magnetic CD31 conjugated microbeads, cells were seeded into six-well plate pre-coated with 0.2% gelatin. On the second day, we changed half of the MCDB 131 medium. On the third day, non-adherent cells were removed by washing with PBS, and changed all medium for each well. As it is shown in Fig.3-1A, on day 1 after first sorting, cells were sporadically attached. The existence of microbeads was shown by their bright dot appearance. On day 3 after first sorting (Fig.3-1 B), some of the cells were grown as a circle, which is the growth characteristic of endothelial cells. But there were still some spindle shaped contaminated fibroblasts can be seen. On day 5 after first sorting (Fig.3-1 C), some of the cells displayed typical "cobblestone" morphology under light microscopy. On day 8 (Fig.3-1 D), the isolated cells were 80% confluent. Cells were detached by EDTA and CD31+ microbeads were used to do the second sorting to increase purity. After that, cells were seeded again in the 6-well plate. On day 10 after second sorting (Fig.3-1 E-F), cells were 80-90% confluent. There were much more "cobblestone" like endothelial cells and less spindle shaped fibroblasts compared with the first sorting observed under light

microscope.

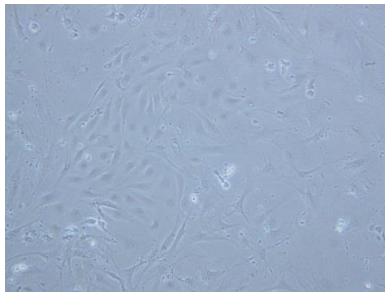
A



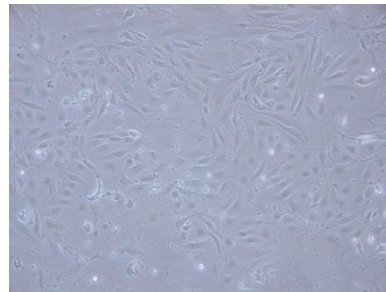
B



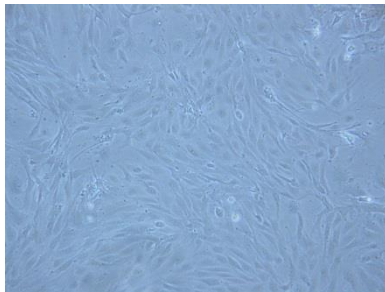
C



D



E



F

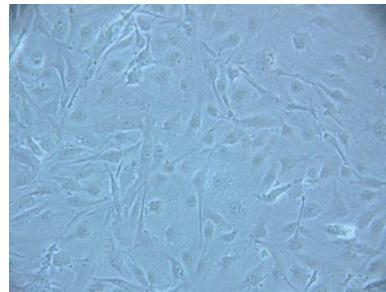


Figure.3-1: Microscopic Morphology of Isolated Microvascular Endothelial Cells from mice lung tissue. Day 1 after first sorting (A), Day 3 after first sorting (B), Day 5 after first sorting (C), Day 8 after first sorting (D), Day 10 after second sorting 40x (E), Day 10 after second sorting 100x (F).

FACS Analysis of PECAM-1 Surface Expression in Cultured MECs

PECAM-1(CD31) is a specific marker for endothelial cells. So when cells got confluent after both first and second sorting, they were stained with anti-rat FITC-conjugated antibody for CD31 and then used for the FACS analysis. Data was analyzed by software FlowJo. As indicated in Fig.3-2 A, there were only 54.1% of CD31+ cells after the first sorting by magnetic beads. But after second sorting, CD31+ cells were increased up to 83.3% (Fig.3-2 B).

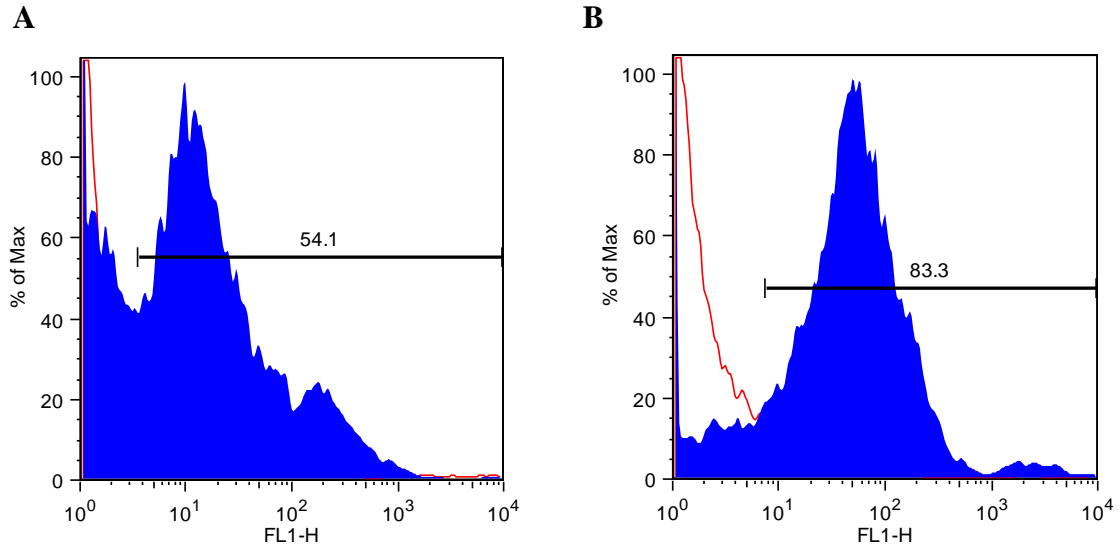


Figure.3-2: Flow Cytometry of PECAM-1(CD31) staining for the isolated Cells.

Isolated cells were subjected to flow cytometry analysis for CD31. A: there is 54.1% CD31+ cell in cultured isolated cells when they become confluent after the first sorting.

B: there is 83.3% CD31+ cell in cultured isolated cells when they become confluent after the second sorting.

Palmitate Dose Dependently Reduces Phosphorylation Of eNOS₁₁₇₇, not eNOS₆₃₃

It is already known that free fatty acids induced adhesion molecules up-regulation is mediated by eNOS-dependent NO reduction. Activity of eNOS is increased by phosphorylation at either Ser₁₁₇₇ or Ser₆₃₃ sites. So we would like to explore which eNOS phosphorylation site is altered by palmitate. Western blot experiment was performed to detect the protein level expression of phosphorylated eNOS Ser₁₁₇₇ and Ser₆₃₃ sites as well as total eNOS.

Palmitate-BSA was added to the cell culture medium for a final concentration analogous to obesity blood palmitate levels to mimic the pathophysiological condition witnessed in vivo. Here, we use concentration from 100 μ M, 200 μ M to 400 μ M, 600 μ M.

Fig.3-3 A and Fig.3-4 A demonstrate that 1 hour palmitate-BSA treatment dose-dependently reduces Ser₁₁₇₇/eNOS phosphorylation in bovine aortic endothelial cell, while it does not influence Ser₆₃₃/eNOS phosphorylation. According to the densitometry analysis (Fig.3-3 B, Fig3-4 B), 600 μ M Palmitate-BSA is most effective in reducing eNOS (P<0.01 vs. BSA) phosphorylation. We then treated cells with 600 μ M palmitate-BSA for different time points from 15 minutes to 150 minutes. But we still failed to see any change in Ser₆₃₃/eNOS phosphorylation (Fig.3-4 C, D).

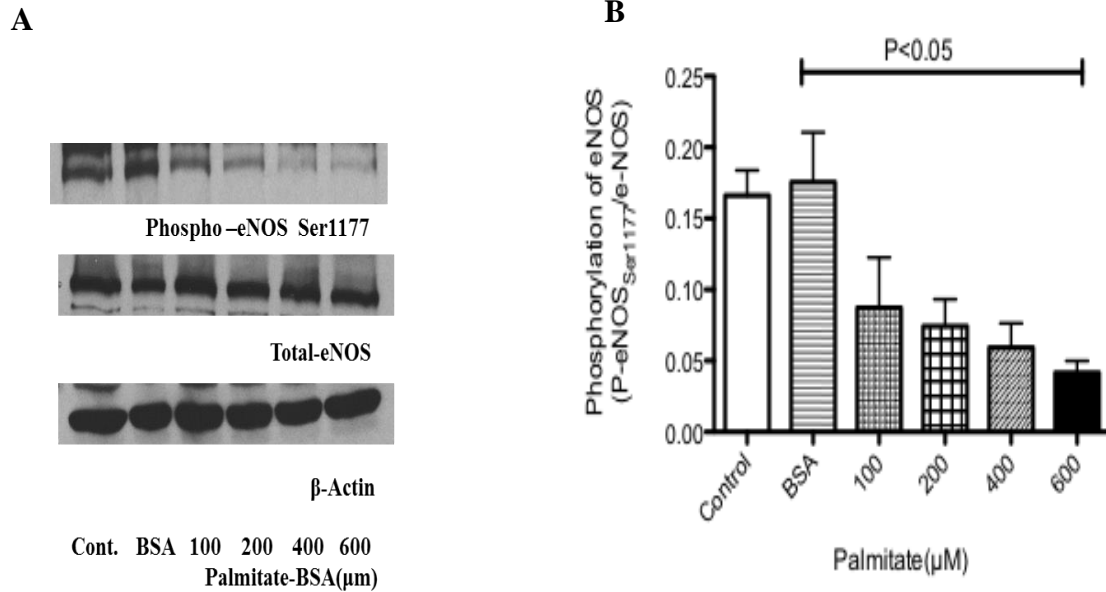


Figure.3-3: Palmitate Dose-dependently Reduces eNOS Ser₁₁₇₇ Phosphorylation.

Bovine Aortic Endothelial Cells (BAECs) were treated with different concentrations of Palmitate-BSA for 60 minutes. Western blot shows that palmitate causes dose-dependently decrease in the phosphorylation of Ser₁₁₇₇/eNOS (A), Bar graphs (B) indicate densitometry quantification of western blot (n=3).

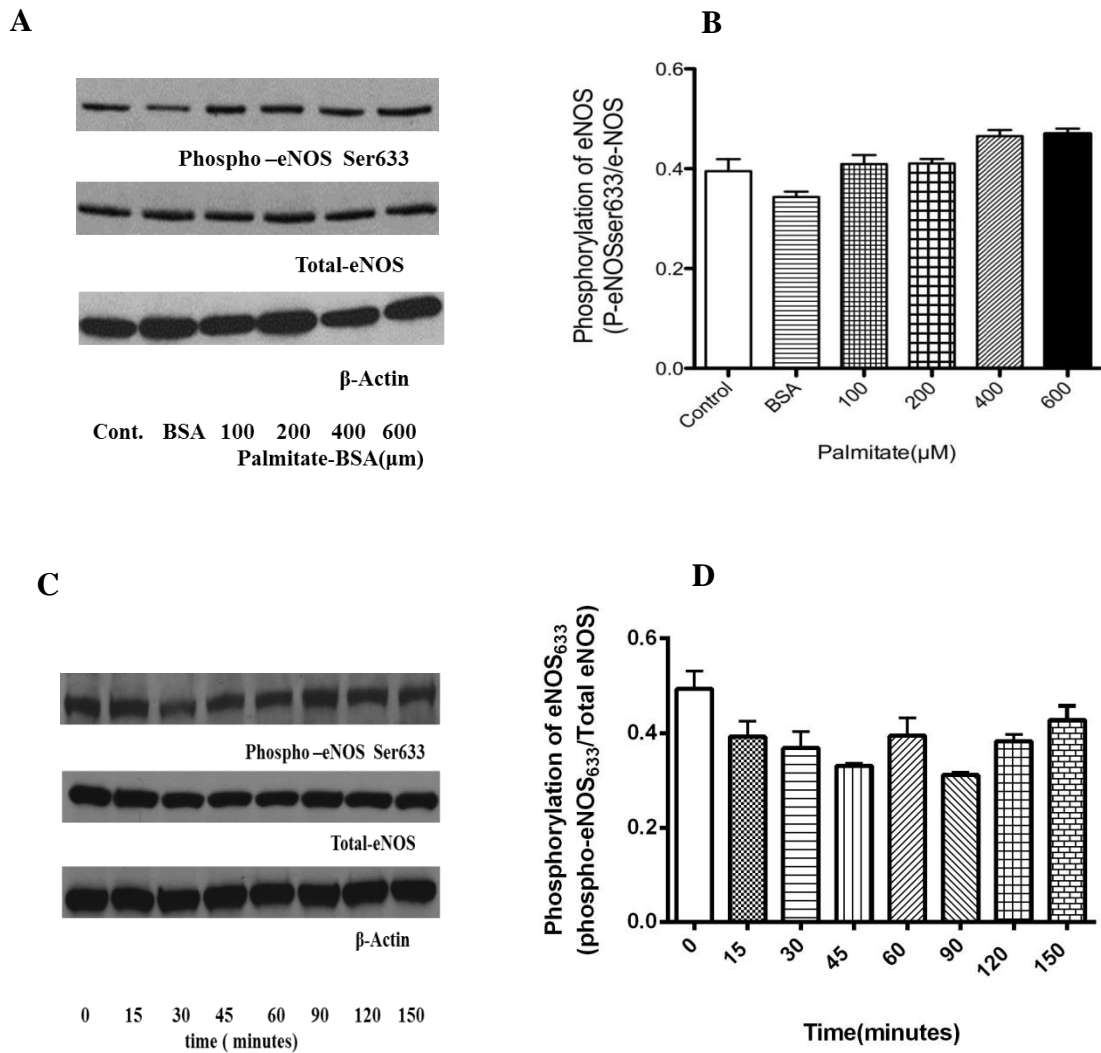


Figure.3-4: Palmitate does not reduce eNOS Ser₆₃₃ phosphorylation. Bovine Aortic Endothelial Cells (BAECs) were treated with different concentrations of Palmitate-BSA for 60 minutes. Western blot shows that palmitate has no influence on Ser₆₃₃/eNOS phosphorylation (A). Bar graphs (B) indicate densitometry quantification of western blot (n=3). BAECs were treated with 600 μ M palmitate-BSA for different time from 0 minutes to 150 minutes and no change was observed in western blot results (C, D).

Palmitate Dose Dependently Reduces AMPK Phosphorylation

AMPK is a very important upstream kinase that could activate eNOS phosphorylation under pathophysiological stimuli. Here, we would like to detect if AMPK phosphorylation could also be altered by palmitate exposure.

Palmitate-BSA was added to the cell culture medium for a final concentration analogous to obesity blood palmitate levels to mimic the pathophysiological condition witnessed in vivo. Here, we used concentration from 100 μ M, 200 μ M to 400 μ M, 600 μ M.

Fig.3-5 A demonstrates that palmitate dose-dependently reduces Thr₁₇₂/AMPK phosphorylation in bovine aortic endothelial cell, while the total AMPK expression was not changed. In addition, according to the densitometry analysis (Fig.3-5 B), 600 μ M Palmitate is most effective in reducing AMPK ($P < 0.01$ vs. BSA) phosphorylation.

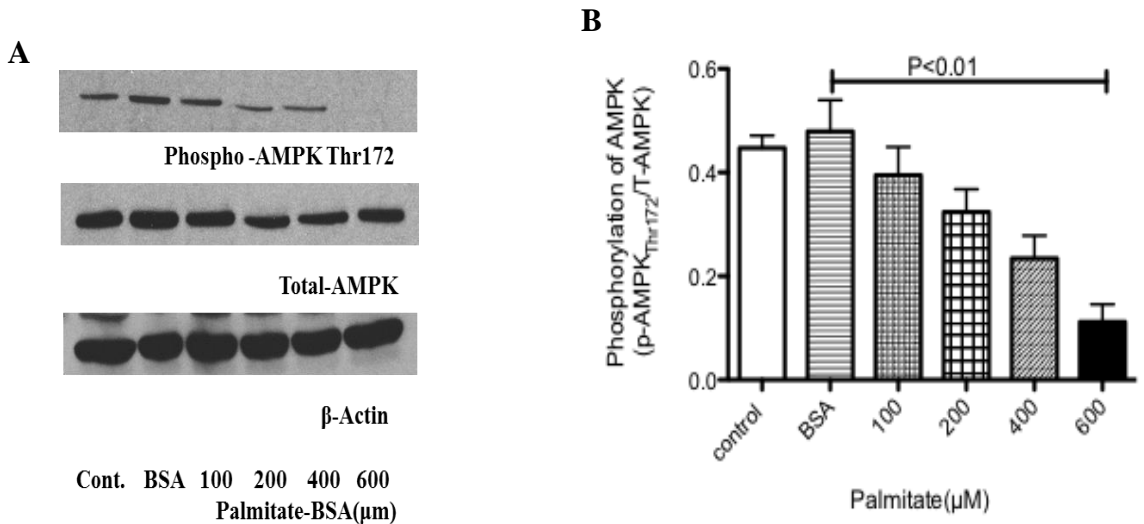


Figure.3-5: Palmitate Dose-dependently Reduces AMPK Phosphorylation. Bovine Aortic Endothelial Cells (BAECs) were treated with different concentrations of Palmitate-BSA for 60 minutes. Western blot shows that palmitate causes dose-dependently decrease in the phosphorylation of Thr₁₇₂/AMPK (A). Bar graphs (B) indicate densitometry quantification of western blot (n= 4).

Calpain Activity is Activated by Palmitate

Calpain has been associated with endothelial dysfunction and calpain inhibition has been implicated in preventing calpain induced endothelial dysfunction. To explore if calpain is activated by palmitate, we measured calpain activity by the specific assay.

Bovine Aortic Endothelial Cells were incubated with 60 μM Succ-LLVY-AMC for 30 minutes at 37°C. Fluorescence (excitation λ 360 nm; emission λ 460 nm) was measured with a Bio-Tek FLx800 microplate fluorescence reader (Bio-Tek, Winooski, VT) for the basal reading. After that, cells were treated with different concentrations of palmitate-BSA (100 μM, 200 μM, 400 μM, 600 μM), measurement was read every 30 minutes until 120 minutes. Fig.3-5 A demonstrates that calpain activity was activated in

30 minutes 400 μ M ($P<0.05$) and 600 μ M ($P<0.01$) palmitate-BSA exposure compared with BSA group. Accordingly, 600 μ M palmitate-BSA is the most effective concentration.

In order to find the earliest time point at which calpain was activated and if this activity could be inhibited by calpain inhibitors, we completed the time point experiments within 30 minutes. Bovine Aortic Endothelial Cells were pretreated with 100 μ M calpain inhibitor Zllal or PD150606 and then incubated with 60 μ M Succ-LLVY-AMC for 30 minutes at 37°C. Fluorescence (excitation λ 360 nm; emission λ 460 nm) was measured with a Bio-Tek FLx800 microplate fluorescence reader (Bio-Tek, Winooski, VT) for the basal reading. After that, cells were treated with 600 μ M Palmitate-BSA for different time points. Calpain activity was calculated as the delta change in fluorescence intensity from the fluorescence reading after palmitate treatment (I_0) to the fluorescence reading after substrate incubation(I_f) according to the equation $\Delta I=I_f-I_0$.

Fig.3-5 B demonstrates that Calpain activity has been activated acutely within 3 minutes after palmitate treatment ($P<0.05$ vs BSA). Then it was continued to be activated until 30 minutes. Both of the two calpain inhibitors ZLLal and PD150606 could prevent calpain activation by palmitate ($P<0.05$ vs Palmitate).

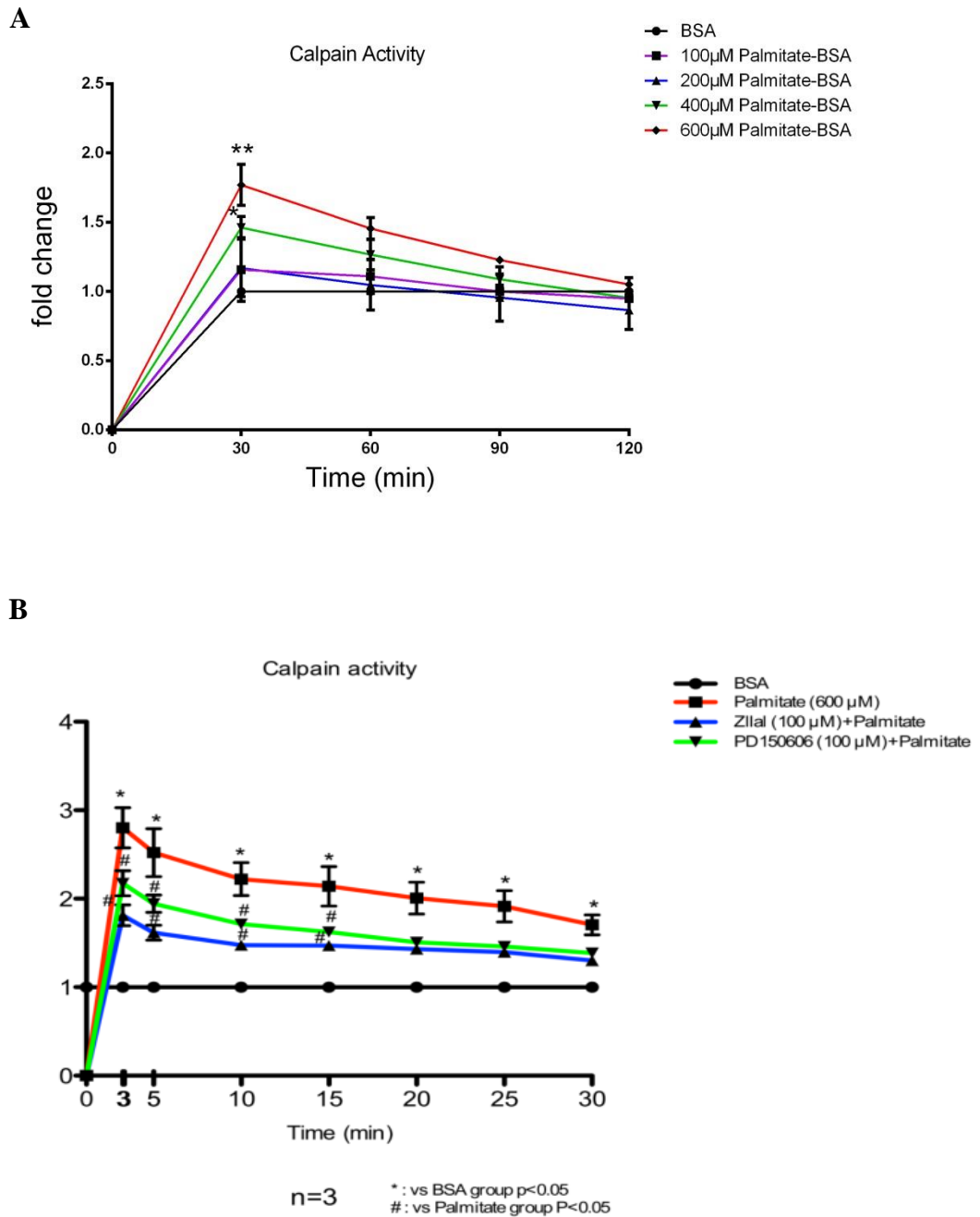


Figure.3-6: Palmitate Acutely Activates Calpain. A: BAECs were treated with 400 µM, 600 µM Palmitate-BSA for 30, 60, 90, 120 minutes. calpain activity was increased by 600 µM palmitate-BSA exposure for 30 minutes (P<0.01). B: BAECs were pretreated with 100µM calpain inhibitor Zllal or PD150606. Then incubate with 600 µM palmitate-BSA

exposure for up to 30 minutes, measurement was read every 5 minutes. Calpain activity has been activated acutely within 3 minutes after palmitate treatment ($P < 0.05$ vs BSA). Then calpain was continued to be activated until 30 minutes. Both of the two calpain inhibitors ZLLal and PD150606 could prevent calpain activation by palmitate ($P < 0.05$ vs Palmitate). $N=3$. ** $P < 0.01$ vs BSA; * $P < 0.05$ vs BSA; # $P < 0.05$ vs Palmitate.

μ -Calpain Instead of m-Calpain Isoform Is Activated by Palmitate

The m and μ -calpain isoform are ubiquitously expressed and thus found in the vascular endothelium. Previous research reported a preferential activation of μ -calpain in Angiotensin II induced endothelial dysfunction (*Scalia R et al., 2011*). Thus, western blot was performed to detect if m and μ -calpain were activated in this scenario.

Rat Heart Microvascular Endothelial Cells were treated with 600 μ M Palmitate-BSA for different time points within 30 minutes. Ionomycin is a direct intracellular calcium activator, thus it could activate the calcium dependent protease calpain. Here it is used as a positive control for calpain activity. We treated cells with 1 μ M ionomycin for 30 minutes. After the treatment, cell lysates were collected for western blot.

Calpain autolyzes the N-terminal end of domain I upon activation. The resulting loss of N-terminal domain antibody detection can be used as a measure of m and μ -calpain activation. As shown in Fig.3-7 A and B, μ -calpain was activated most significantly after palmitate treatment for 5 minutes ($P < 0.01$ vs. BSA) and 10 minutes ($P < 0.05$ vs. BSA). However, the same activity was not observed in m-calpain (Fig.3-8 A and B).

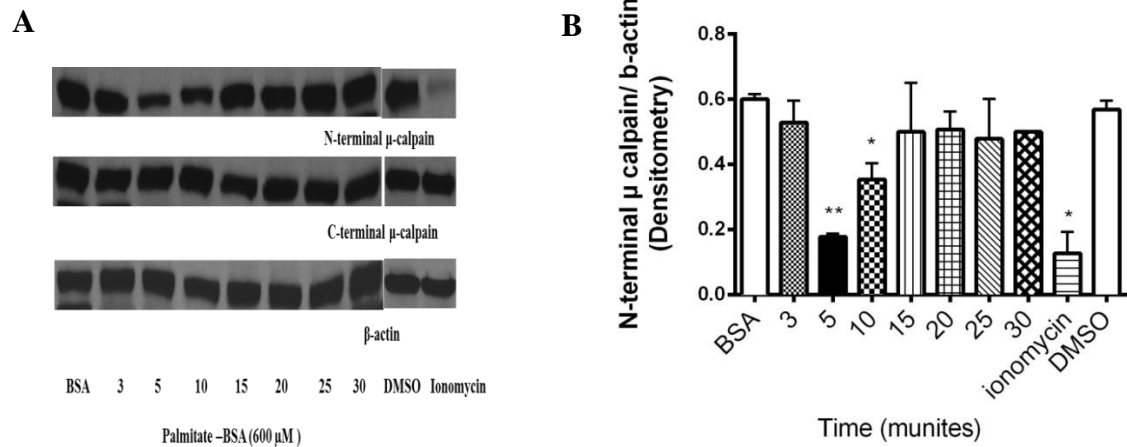


Figure.3-7: μ -Calpain Isoform was Activated by Palmitate. **A:** Rat Heart Microvascular Endothelial Cells were treated with 600 μ M Palmitate-BSA for different time points and also with 1 μ M ionomycin for 30 minutes. Expression of m and μ -Calpain N-terminal and C-terminal were analyzed by immunoblotting. **B:** Bar graph indicates densitometry quantification of western blot (n=2). (**P<0.01, *P<0.05. All the time points group vs BSA, ionomycin vs DMSO).

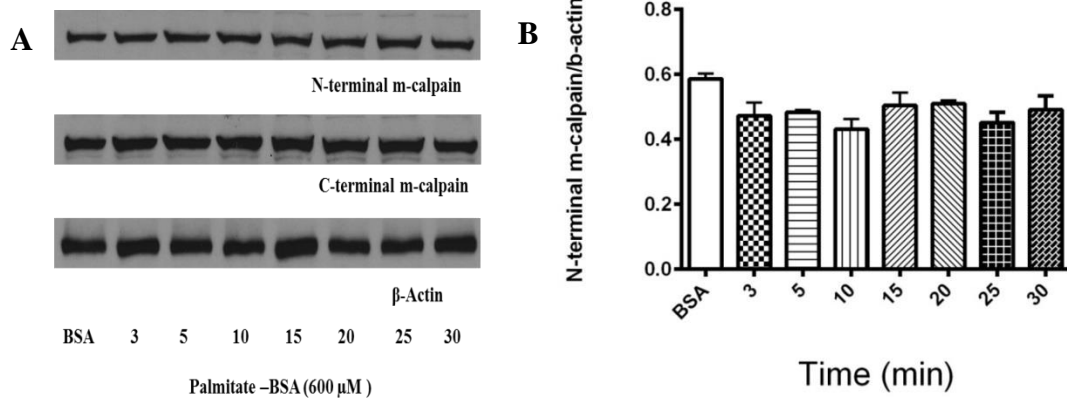


Figure.3-8: m-Calpain Isoform was not activated by Palmitate. **A:** Rat Heart Microvascular Endothelial Cells were treated with 600 μ M Palmitate-BSA for different time points. Expression of m and m-Calpain N-terminal and C-terminal were analyzed by immunoblotting. **B:** Bar graph indicates densitometry quantification of western blot (n=2) (All the time points group vs BSA).

CHAPTER 4

DISCUSSION

Overview

Previous literatures show that palmitate exposure activates leukocyte recruitment through up-regulation of endothelial adhesion molecules via reduced eNOS derived eNO production (*Rizzo NO et al., 2010; Davenpeck KL et al., 1994; Ahluwalia A et al., 2004*). Overall, this thesis has two main contributions in research of this area: 1. A stable, efficient primary microvascular endothelial cell isolation technics by microbeads sorting was established, which provides a meaningful source for *in vitro* endothelial cell study. 2. The collected data implied a potential role of the AMPK, eNOS signaling in palmitate induced adhesion molecules up-regulation. Additionally, this thesis is also the first one to demonstrate that endothelial calpain, a calcium dependent protease, specifically μ -calpain isoform is activated by palmitate (Fig.4-1). The major dedication of this study is to enhance the knowledge of vascular cell signal transductions in SFAs induced endothelial dysfunction. It may also lead to a new way of clinically treating obesity related vascular diseases.

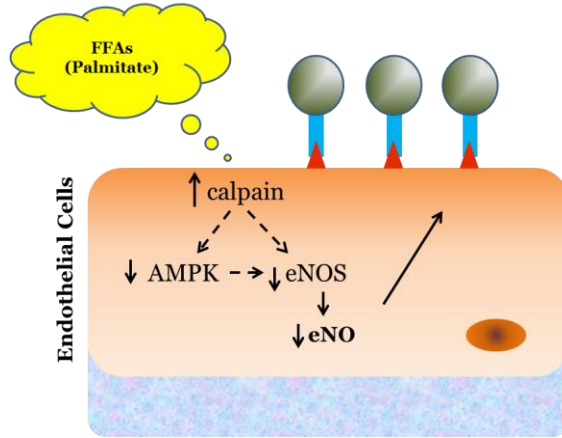


Figure.4-1: Schematic Illustration of Palmitate Induced Endothelial Adhesion Molecule Up-Regulation. Excess palmitate may cause loss of basal eNO levels and up-regulation of adhesion molecules via down-regulation of AMPK and eNOS activity. More interestingly, palmitate also activates endothelial calpain. FFA: Free Fatty Acids; AMPK: AMP-activated protein kinase; eNOS: endothelial Nitric oxide synthases; eNO: endothelial nitric oxide.

Discussion

In recent years, isolation and purification of endothelial cells for culture has been gaining more and more interest. However, these techniques are all prone to get contaminated cell types (i.e. fibroblasts). In this thesis, endothelial cells were isolated based on Antibody-coated magnetic beads. There are several advantages of this technique. First, compared with traditional cell sorting by flow cytometry, the proposed technique adds less mechanical damage to the cells during sorting. Therefore cells could still be used in future experiments without any characteristic change. Second, with specific antibody binding, it is possible to get highly purified endothelial cells and repeated beads sorting which could enhance the purity. In this thesis, after the first beads sorting, 54.1%

CD31+ cells was obtained and after the second sorting, it was elevated up to 83.3%. Finally, the proposed process is more economical and easier to handle. A lot of trouble shooting has been done during the development of the proposed technique and it is still possible that the purity will be further enhanced after more practice.

There are several factors in the protocol that, we believe, contribute to the successful isolation of primary microvascular endothelial cells from mice lung tissue and enhance the overall purity of endothelial cells among those isolated cells during culturing. The first is the use of lungs from 3 week-old mice rather than adult rodents. Although we could isolate MECs from adult mice with the same protocol, the yields of cells were much less and it takes a much longer time for those cells to grow confluent. This is because cells from young animals have a higher proliferation potential under culture *in vitro*. Another key point in our protocol is the method we used to get microvascular endothelial cells, preventing large vessel endothelial cell contamination. When we get lung tissues from mice, we avoid pulmonary hilus where large vessels exist, and cut small peripheral sections of lungs. In addition, we also removed large vessels every time before mince sample. A second important feature in our protocol is the way we prepare for single cell suspension during primary cell isolation. There are four steps in preparation of single cell suspension from fresh tissue sample, including mince, digest, sieve and RBC lysis. Specifically, tissues were initially minced by surgical blades for 20 to 30 minutes until become $<1 \text{ mm}^3$ pieces. After that, we use the mixture of collagenase, dispase and DNaseI solution to digest the tissue properly for 1 hour with agitation every 5 minutes, then sieve the sample through different size of strainers (40um and 100um). The purpose is to decrease the amount of cell clumps and cell debris. RBC lysis buffer was used to

exclude large amount of RBCs in the sample. Another critical element in our protocol is the method we developed to get rid of fibroblasts contamination. On the one hand, we sorted cells with CD31 antibody conjugated microbeads twice to enhance endothelial cell purity. We also used 0.2% gelatin pre-coated plates to seed the cells for helping endothelial cells better attach to the bottom of plate. On the other hand, since fibroblasts grow faster than endothelial cells, they need more serum to extract nutrition. We tried three different serum concentrations: 2%, 5% and 10% to culture cells immediately after first sorting. It was found that using a 5% serum medium to culture will limit the growth of fibroblasts and get a better endothelial cells survival rate. So it is concluded that 5% serum medium can be the perfect environment that could both provide nutrition for endothelial cells and inhibit the fast growth of fibroblasts. While, after removing fibroblast, it is recommended to use a higher percentage of FBS in medium, promoting growth of endothelial cells. The last significant factor is that we used 1 μ M EDTA instead of trypsin to detach cultured cells for either second sorting or FACS analysis. Due to the sensitivity of mouse CD31 to be cleaved by trypsin, we alternatively chose 1 μ M EDTA solution to detach cells, which prevented damaging CD31 molecule on cell surface.

Dietary FFAs can be classified into three categories: SFAs, MUFAs and PUFAs. Among all three categories, SFAs, raised from the consumption of dairy, animal products and products extracted from certain vegetable oils such as coconut oils and palm oils is the most abundant ingredient in a typical American diet. The length of SFAs' molecule chain varies from 4 to 20 carbon atoms and the molecule with 12-18 carbon atoms is predominant in the diet. The major SFAs in most human diets are palmitic acid (16:0)

(Katan MB, et al., 1994). Palmitic acid is also a commonly accepted model for the study of SFAs associated disease. Thus, in order to observe the specific effect of FFAs in endothelial dysfunction, this thesis is focused on palmitate.

Ser₁₁₇₇ appears to be the most important of the regulatory eNOS phosphorylation sites. Its phosphorylation in response to a list of stimuli including mechanical factors like shear stress, humoral factors such as bradykinin, insulin, VEGF and pharmacological activator such as statins. Furthermore, many kinases have been implicated in the regulation of eNOS Ser₁₁₇₇ phosphorylation which are AMPK, Akt, PKA, CaMKII, PP2A et al. Phosphorylation of eNOS Ser₁₁₇₇ site mediated by Ca²⁺/CaM binding (*Chen ZP et al, 1999; Michell BJ et al, 2002*) and this eNOS activation can also happen at resting level of [Ca²⁺] (*Montagnani M et al, 2001*). Additionally, eNOS activity is also increased by phosphorylation of another site, Ser₆₃₃. Several activators such as shear stress, bradykinin, VEGF and statins that increase phosphorylation of Ser₁₁₇₇ also phosphorylate the Ser₆₃₃ site. But the phosphorylation of eNOS Ser₆₃₃ was found to be slower than Ser₁₁₇₇. Moreover, stimulation of eNOS Ser₆₃₃ phosphorylation does not require an increase in intracellular Ca²⁺ (*Boo YC et al, 2003*). Overall, it is suggested that eNOS Ser₆₃₃ is important in either activating eNOS without Ca²⁺ influx, or maintaining increased eNOS activity by Ca²⁺ influx and/or Ser₁₁₇₇ phosphorylation. Data in this thesis showed that phosphorylation of eNOS Ser₁₁₇₇ instead of Ser₆₃₃ was reduced by a 1-hour palmitate treatment. We then detect Ser₆₃₃ phosphorylation after palmitate treatment time from 0 to 150 minutes, but no difference was found. Our results suggest that palmitate reduces eNOS activity through decreasing the phosphorylation of eNOS₁₁₇₇, not eNOS₆₃₃. The possible reason could be the influx of Ca²⁺ by palmitate. Moreover, it

has been confirmed that palmitate dose-dependently reduces the phosphorylation of AMPK thr₁₇₂ and eNOS₁₁₇₇ from western blot. However, whether or not AMPK exhibit a causal effect in Palmitate induced reduction of eNOS phosphorylation still deserves further investigation.

The initial evidence, that calpain playing a role in the endothelial dysfunction of diabetes, is from paper published by our laboratory. Specifically, acute hyperglycemia (*Stalker TJ et al., 2003*) and type 2 diabetes (*Stalker TJ et al., 2005*) were found to up-regulate calpain activity which then cause endothelial dysfunction. Right now, investigation has come into other metabolic disturbances, such as hyperlipidemia (*Dong Y et al., 2008*), causing calpain activation. Studies showed that the conditioned medium from palmitate-treated astrocytes elevated the calcium level in primary cortical neurons and increased calpain activity (*Li Qi Liu et al., 2013*). Based on the above evidence, we sought to study vascular calpain in a setting of free fatty acids exposure.

The thesis has firstly demonstrated that endothelial calpain was activated at a very early stage by palmitate stimulation, within 3 to 5 minutes by specific calpain activity assay (Fig.3-5). This can be explained by the mechanism of calpain intracellular activation. Calpain is a calcium dependent cysteine protease. Increased cytoplasm calcium influx quickly binds with the regulatory site on calpain subunit and activates calpain. Upon protease activation, calpain translocate into the cell membrane to proteolyze other downstream molecules. This process can happen in a few minutes. According to our data, endothelial cells were treated by palmitate with different time points from 30 minutes to 2 hours. Calpain activity was activated in 30 minutes, after that its activity dropped dramatically (Fig.3-5). It is found in this thesis that palmitate

selectively activates the μ -calpain isoform, but not the m-calpain isoform. We think this result is compatible with the knowledge that only the micromole Ca^{2+} concentrations required by autolyzed μ -calpain are within the physiological range, whereas the millimole Ca^{2+} concentrations required by m-calpain are much higher than those found in living cells.

When studying kinases and proteases, non-specific effects of pharmacological inhibitors are always a particular concern. Thus, in this thesis, experiments were repeated on the inhibition of calpain with more than one pharmacological inhibitor. Administration of ZLLal or PD150606 similarly partially restored calpain activation and ZLLal seems that has a better effect on calpain inhibition (Fig.3-5). The more effective inhibition of ZLLal could be explained by its direct binding with the calpain catalytic site, while PD150606 indirectly interacts with Ca^{2+} binding site. The partial instead of complete inhibition effect could attribute to the non-specific effects of pharmacological drugs. Thus, it can be concluded that calpain was properly targeted with the pharmacological inhibitors in this thesis.

We already demonstrated that palmitate activates endothelial calpain, reduces eNOS activity. Averna M et al. proved in their paper that eNOS is a sensitive calpain substrate and the ratios of chaperon heat shock protein HSP90 / nitric oxide synthase (NOS) in the cell determines the digestion of eNOS (Averna M et al, 2008). In the absence of Ca^{2+} , HSP90 forms complexes with NOS or with calpain. When Ca^{2+} is present, a ternary complex containing the three proteins is produced. In this associated state, HSP90 and NOS are almost completely resistant to calpain digestion. The possible reason for this is the reduction in the catalytic efficiency of the protease. Thus, the

recruitment of calpain in the HSP90–NOS complexes reduces the extent of the proteolysis of these two proteins. Because of the low level of HSP90 in endothelial cells, it is possible that eNOS is digested by activated endothelial calpain.

Conclusions

It is found in this thesis that palmitate exposure dose dependently reduces endothelial AMPK thr₁₇₂ and eNOS ser₁₁₇₇ phosphorylation by western blot. Moreover, this study also showed that endothelial calpain, a calcium dependent protease associated with endothelial dysfunction, was activated by palmitate, specifically its μ -calpain isoform. Altogether, these data suggested that a significant role of calpain, the calcium sensitive protease, as a key mediator of palmitate induced endothelial dysfunction and indicate both AMPK and eNOS₁₁₇₇ phosphorylation site contribute to this pathological process. Further investigations are still needed to explore connections among those molecules. Thus, these findings suggest a novel future therapy that may involve a multi-drug regimen targeting calpain to prevent obesity microvascular complications. Another important novelty of this thesis is established methods of primary microcirculation endothelial cells isolation from fresh mice tissue, which is very significant in studying mechanisms of leukocyte infiltration during inflammation in specific tissue.

Future Directions

First of all, since the protocol to isolate primary endothelial cells from lung tissue has already been established, it is very meaningful to use the similar adjusted technics and get microvascular endothelial cells from adipose tissue, especially visceral fat which should be a good cell source to study adipose tissue inflammation *in vitro*.

Preliminary results in this thesis showed that eNOS, AMPK involved in palmitate induced endothelial dysfunction, but more experiments are needed to further confirm this finding. Firstly, both L-NAME (eNOS inhibitor), compound C (AMPK inhibitors) and NO, AICAR (AMPK activators) should be added to check if the same effects caused by palmitate treatment would either be inhibited or enhanced. Secondly, isolated microvascular endothelial cells are recommended to be used and the same effects should be observed. At last, to prove this finding, more animal experiments are necessary to be done. Use western blot and immunohistochemistry technics to check the protein expression in animal tissues.

Since three separated endothelial molecules involved in palmitate pathway have already been discovered, it is necessary to prove the connections between these molecules and their relation to adhesion molecules expression. There are two appealing but still unproved hypothesis from current study could be conducted on how palmitate activates calpain, then down-regulates eNOS activity and finally reduces eNO production. Recent papers also suggested that chronic high cytosolic calcium can decrease AICAR-induced AMPK activity via calcium/calmodulin activated protein kinase II signaling cascade (*S.Park et al., 2011*). Some investigation proved the fact that calpain plays a vital role in muscle protein turnover and that GLUT4, AMPK, and CAMKII are all calpain substrates (*Otani K et al., 2006*). Since both AMPK and eNOS are calpain substrates and eNOS is also downstream activated by AMPK, calpain could either decrease eNOS activity through inhibit AMPK activity or may directly reduce eNOS activity. More experiments should be performed to explore those possibilities. Calpain and AMPK specific inhibitors and activators are recommended to be used in the future study to prove

the hypothesis. It is also reasonable to use μ -calpain knock-out mice to prove that if μ -calpain isoform is part of this phenomenon *in vivo*.

REFERENCES CITED

1. Ahluwalia A, Foster P, Scotland RS, McLean PG, Mathur A, Perretti M, et al. Antiinflammatory activity of soluble guanylate cyclase: cGMP-dependent down-regulation of P-selectin expression and leukocyte recruitment. *Proc Natl Acad Sci USA*.2004; 101 (5):1386 – 91.
2. Averna M, Stifanese R, De Tullio R, Passalacqua M, Salamino F, Pontremoli S, Melloni E. Functional role of HSP90 complexes with endothelial nitric-oxide synthase (eNOS) and calpain on nitric oxide generation in endothelial cells. *J Biol Chem*. 2008 Oct 24;283(43):29069-76.
3. Bauer KW, Hearst MO, Earnest AA, French SA, Oakes JM, Harnack LJ. Energy content of U.S. Fast-food restaurant offerings: 14-year trends. *Am J Prev Med*. 2012; 43(5):490 – 7.
4. Bauer PM, Fulton D, Boo YC, Sorescu GP, Kemp BE, Jo H, Sessa WC. Compensatory phosphorylation and protein-protein interactions revealed by loss of function and gain of function mutants of multiple serine phosphorylation sites in endothelial nitric-oxide synthase. *J Biol Chem*. 2003; 278: 14841–14849.
5. Bays H, Mandarino L, DeFronzo RA. Role of the adipocyte, free fatty acids, and ectopic fat in pathogenesis of type 2 diabetes mellitus: peroxisomal proliferator-activated receptor agonists provide a rational therapeutic approach. *J Clin Endocrinol Metab* 2004; 89:463–78.
6. Berg AH, Scherer PE. Adipose tissue, inflammation, and cardiovascular disease. *Circ Res*. 2005 May 13; 96(9):939-49.

7. Boivin A, Brochu G, Marceau S, Marceau P, Hould FS, Tchernof A. Regional differences in adipose tissue metabolism in obese men. *Metabolism*. 2007; 56(4):533 – 40.
8. Bonnet M, Leroux C, Faulconnier Y, et al. Lipoprotein lipase activity and mRNA are up-regulated by refeeding in adipose tissue and cardiac muscle of sheep. *J Nutr*. 2000 Apr;130 (4):749-56.
9. Boo YC, Hwang J, Sykes M, Michell BJ, Kemp BE, Lum H, Jo H. Shear stress stimulates phosphorylation of endothelial nitric-oxide synthase at Ser1179 by Akt-independent mechanisms: role of protein kinase A. *J Biol Chem*. 2002; 277: 3388–3396.
10. Boo YC, Sorescu GP, Bauer PM, Fulton D, Kemp BE, Harrison DG, et al. Endothelial NO synthase phosphorylated at SER635 produces NO without requiring intracellular calcium increase. *Free Radic Biol Med* 2003; 35 (7):729 – 41.
11. Bosanská L, Michalský D. The influence of obesity and different fat depots on adipose tissue gene expression and protein levels of cell adhesion molecules. *Physiol Res*. 2010;59(1):79-88.
12. Cacicedo JM, Yagihashi N, Keaney JF Jr, Ruderman NB, Ido Y. AMPK inhibits fatty acid-induced increases in NF- κ B transactivation in cultured human umbilical vein endothelial cells. *Biochem Biophys Res Commun*. 2004; 324:1204–1209.

13. Chen H, Montagnani M, Funahashi T, Shimomura I, Quon MJ. Adiponectin stimulates production of nitric oxide in vascular endothelial cells. *J Biol Chem.* 2003;278: 45021–45026.
14. Dagher Z, Ruderman N, Tornheim K, Ido Y. The effect of AMP-activated protein kinase and its activator AICAR on the metabolism of human umbilical vein endothelial cells. *Biochem Biophys Res Commun.* 1999; 265: 112–115.
15. Davenpeck KL, Gauthier TW, Lefer AM. Inhibition of endothelial-derived nitric oxide promotes P-selectin expression and actions in the rat microcirculation. *Gastroenterology.* 1994; 107 (4):1050 – 8.
16. Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. *Circulation.* 2004 Jun 15; 109.
17. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature.* 1999; 399: 601–605.
18. Djoussé L, Benkeser D, Arnold A, Kizer JR, et al. Plasma free fatty acids and risk of heart failure: the Cardiovascular Health Study. *Circ Heart Fail.* 2013 Sep 1;6(5):964-9.
19. Doege H, Stahl A. Protein-mediated fatty acid uptake: novel insights from in vivo models. *Physiology (Bethesda).* 2006; 21: 259–268.
20. Dong Y, Wu Y, Wu M, Wang S, Zhang J, Xie Z, Xu J, Song P, Wilson K, Zhao Z, Lyons T, and Zou MH. Activation of Protease Calpain by Oxidized and Glycated LDL Increases the Degradation of Endothelial Nitric Oxide Synthase. *J Cell Mol Med* 2008.

21. Egger G, Dixon J. Inflammatory effects of nutritional stimuli: further support for the need for a big picture approach to tackling obesity and chronic disease. *Obes Rev.* 2010 Feb;11(2):137-49.
22. F. Addabbo, M. Montagnani, M.S. Goligorsky, Mitochondria and reactive oxygen species, *Hypertension* 2009; 53: 885–892.
23. Frohnert BI, Bernlohr DA. Regulation of fatty acid transporters in mammalian cells. *Prog Lipid Res* 2000; 39: 83–107.
24. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature.*1999;399: 597–601.
25. Furuhashi M1, Fucho R, Görgün CZ, Tuncman G, Cao H, Hotamisligil GS. Adipocyte/macrophage fatty acid-binding proteins contribute to metabolic deterioration through actions in both macrophages and adipocytes in mice. *J Clin Invest.* 2008 Jul; 118(7):2640-50.
26. Geng J. G. Interaction of vascular endothelial cells with leukocytes, platelets and cancer cells in inflammation, thrombosis and cancer growth and metastasis. *Acta Pharmacol Sin.*2003; 24:1297–1300.
27. Gerald M. Reaven. Measurement of Plasma Glucose, Free Fatty Acid, Lactate, and Insulin for 24 h in Patients with NIDDM. *Diabetes.*1988, Aug; Vol. 37.
28. Gersh I, Still MA. Blood vessels in fat tissue: Relation to problems of gas exchange. *J Exp Med.* 1945; 81(2):219 – 32.
29. Goll DE, Thompson VF, Li H, Wei W, and Cong J. The calpain system. *Physiol Rev.* 2003; 83: 731-801.

30. Haffner SM. Abdominal adiposity and cardio-metabolic risk: do we have all the answers? *Am J Med.* 2007; 120, S10–S16; discussion S16-17.
31. Hajri T, Abumrad NA. Fatty acid transport across membranes: relevance to nutrition and metabolic pathology. *Annu Rev Nutr.* 2002; 22: 383–415.
32. Hall WL. Dietary saturated and unsaturated fats as determinants of blood pressure and vascular function. *Nutr Res Rev.* 2009 Jun; 22(1):18-38.
33. Hamilton JA, Kamp F. How are free fatty acids transported in membranes? Is it by proteins or by free diffusion through the lipids? *Diabetes* 1999;48: 2255–2269.
34. Harris MB, Blackstone MA, Sood SG, Li C, Goolsby JM, Venema VJ, Kemp BE, Venema RC. Acute activation and phosphorylation of endothelial nitric oxide synthase by HMG-CoA reductase inhibitors. *Am J Physiol Heart Circ Physiol.* 2004;287: 560–566.
35. Hattori Y, Nakano Y, Hattori S, Tomizawa A, Inukai K, Kasai K. High molecular weight adiponectin activates AMPK and suppresses cytokine-induced NF- κ B activation in vascular endothelial cells. *FEBS Lett.* 2008; 582: 1724.
36. Hewett PW, Murray JC. Human lung microvessel endothelial cells: isolation, culture, and characterization. *Microvasc Res.* 1993 Jul; 46 (1):89-102.
37. Hirose, T. Tanikawa, H. Mori, Y. Okada, Y. Tanaka, Advanced glycation endproducts increase endothelial permeability through the RAGE/Rho signaling pathway, *FEBS Lett.* 2010; 584: 61–66.

38. Ichimura H, Parthasarathi K, Quadri S, Issekutz AC, Bhattacharya J. Mechano-oxidative coupling by mitochondria induces pro-inflammatory responses in lung venular capillaries. *J Clin Invest.* 2003; 111(5):691 – 9.
39. Ikeda Y, Young LH, and Lefer AM. Attenuation of neutrophil-mediated myocardial ischemia-reperfusion injury by a calpain inhibitor. *American journal of physiology.* 2002; 282: H1421-1426.
40. Ioka RX, Kang MJ, Kamiyama S, Kim DH, Magoori K, Kamataki A et al. Expression cloning and characterization of a novel glycosylphosphatidylinositol anchored high density lipoprotein-binding protein, GPI-HBP1. *J Biol Chem* 2003; 278:7344–7349.
41. Kampf JP, Parmley D, Kleinfeld AM. Free fatty acid transport across adipocytes is mediated by an unknown membrane protein pump. *Am J Physiol Endocrinol Metab* 2007; 293:E1207–E1214.
42. Katan MB, Zock PL, Mensink RP. Effects of fats and fatty acids on blood lipids in humans: an overview. *Am J Clin Nutr.* 1994 Dec; 60 (6 Suppl):1017S-1022S.
43. Kim JY, van de Wall E, Laplante M, Azzara A, Trujillo ME, Hofmann SM, Schraw T, Durand JL, Li H, Li G et al. Obesity-associated improvements in metabolic profile through expansion of adipose tissue. *J Clin Invest.* 2007; 117, 2621–2637.
44. Krieglstein CF, Granger DN. Adhesion molecules and their role in vascular disease. *Am J Hypertens.* 2001; 14: 44S – 54.
45. Kudo N, Barr AJ, Barr RL, Desai S, Lopaschuk GD. High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in

- malonyl-CoA levels due to an increase in 5-AMP-activated protein kinase inhibition of acetyl-CoA carboxylase. *J Biol Chem.*1995; 270: 17513–17520.
46. Lafontan M. Advances in adipose tissue metabolism. *Int J Obes (Lond)*. 2008 Dec; 32 Suppl 7:S39-51.
 47. Last, Allen R.; Wilson, Stephen A. "Low-Carbohydrate Diets". *American Family Physician* 2006; 73 (11): 1942–8.
 48. Li Qi Liu, Rebecca Martin, Garrett Kohler, Christina Chan. Palmitate induces transcriptional regulation of BACE1 and presenilin by STAT3 in neurons mediated by astrocytes. *Exp. Neurol.* 2013.
 49. Lindholm CR, Ertel RL, Bauwens JD, Schmuck EG, Mulligan JD, Saupe KW. A high-fat diet decreases AMPK activity in multiple tissues in the absence of hyperglycemia or systemic inflammation in rats. *J Physiol Biochem.* 2012.
 50. M. Félétou, P.M. Vanhoutte, Endothelial dysfunction: a multifaceted disorder (the Wiggers award lecture), *Am. J. Physiol. Heart Circ. Physiol.* 2006; 291: H985–H1002.
 51. M. Félétou, The Endothelium: Part 1: Multiple Functions of the Endothelial Cells—Focus on Endothelium-derived Vasoactive Mediators, Morgan & Claypool Life Sciences, San Rafael (CA), 2011.
 52. M.H. Laughlin, S.C. Newcomer, S.B. Bender, Importance of hemodynamic forces as signals for exercise-induced changes in endothelial cell phenotype. *J. Appl. Physiol.* 2008; 104: 588–600.
 53. Margaret F, Gregor and Gokhan S. Hotamisligil. Inflammatory Mechanisms in Obesity. *Ann.Rev.Immunol.*2011; 29: 415-45.

54. Mathew M, Tay E, Cusi K. Elevated plasma free fatty acids increase cardiovascular risk by inducing plasma biomarkers of endothelial activation, myeloperoxidase and PAI-1 in healthy subjects. *Cardiovasc Diabetol*. 2010 Feb 16;9:9.
55. Matsushita K, Morrell CN, Cambien B, Yang SX, Yamakuchi M, Bao C, et al. Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. *Cell*. 2003;115 (2):139 – 50.
56. Michell BJ et al. Identification of Regulatory Sites of Phosphorylation of the Bovine Endothelial Nitric-oxide Synthase at Serine 617 and Serine 635. *J Biol Chem*. 2002 Nov 1; 277(44):42344-51.
57. Michell BJ, Chen ZP, Tiganis T, Stapleton D, Katsis F, Power DA, Sim AT, Kemp BE. Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase. *J Biol Chem*. 2001; 276: 17625–17628.
58. Michell BJ, Harris MB, Chen ZP, Ju H, Venema VJ, Blackstone MA, Huang W, Venema RC, Kemp BE. Identification of regulatory sites of phosphorylation of the bovine endothelial nitric-oxide synthase at serine 617 and serine 635. *J Biol Chem*. 2001; 277: 42344–42351.
59. Monica Averna, Roberto Stifanese, Roberta De Tullio, Mario Passalacqua, Franca Salamino, Sandro Pontremoli and Edon Melloni. Functional Role of HSP90 Complexes with Endothelial Nitric-oxide Synthase (eNOS) and Calpain on Nitric Oxide Generation in Endothelial Cells. *J. Biol. Chem*. 2008; 283:29069-29076.

60. Montagnani M. et al. Insulin-stimulated Activation of eNOS Is Independent of Ca²⁺ but Requires Phosphorylation by Akt. at Ser 1179. *J Biol Chem.* 2001 Aug 10; 276(32):30392-8.
61. Mount PF, Kemp BE, Power DA. Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation. *J Mol Cell Cardiol.*2007;42: 271–279.
62. Mugabo Y, Mukaneza Y, Renier G. Palmitate induces C-reactive protein expression in human aortic endothelial cells. Relevance to fatty acid-induced endothelial dysfunction. *Metab Clin Exp.* 2011; 60(5):640 – 8.
63. Okayasu T, Tomizawa A, Suzuki K, Manaka K, Hattori Y. PPAR activators upregulate eNOS activity and inhibit cytokine-induced NF- κ B activation through AMP-activated protein kinase activation. *Life Sci.* 2008; 82: 884–891.
64. Oram JF, Bornfeldt KE. Direct effects of long-chain nonesterified fatty acids on vascular cells and their relevance to macrovascular complications of diabetes. *Front Biosci.*2004; 9:1240–53.
65. Otani K, Polonsky KS, Holloszy JO, Han DH. Inhibition of calpain results in impaired contraction-stimulated GLUT4 translocation in skeletal muscle. *Am J Physiol Endocrinol Metab.* 2006 Sep; 291(3):E544-8.
66. P. Vallance, Importance of asymmetrical dimethylarginine in cardiovascular risk, *Lancet* 2001; 358: 2096–2097.
67. P.O. Bonetti, L.O. Lerman, A. Lerman, Endothelial dysfunction: a marker of atherosclerotic risk, *Arterioscler. Thromb. Vasc. Biol.* 2003; 23: 168–175.

68. Pilz S, März W. Free fatty acids as a cardiovascular risk factor. *Clin Chem Lab Med.* 2008;46 (4):429-34.
69. Rizzo NO, Maloney E, Pham M, Luttrell I, Wessells H, Tateya S, Daum G, Handa P, Schwartz MW, Kim F. Reduced NO-cGMP signaling contributes to vascular inflammation and insulin resistance induced by high-fat feeding. *Arterioscler Thromb Vasc Biol.* 2010 Apr; 30 (4):758-65.
70. Roth Flach RJ, Matevossian A, Akie TE. β 3-Adrenergic receptor stimulation induces E-selectin-mediated adipose tissue inflammation. *J Biol Chem.* 2013 Jan 25;288(4):2882-92.
71. S. Moncada, E.A. Higgs, Nitric oxide and the vascular endothelium, *Handb. Exp. Pharmacol.* 2006; 176: 213–254.
72. S. Taddei, L. Ghiadoni, A. Viridis, D. Versari, A. Salvetti, Mechanisms of endothelial dysfunction: clinical significance and preventive non-pharmacological therapeutic strategies, *Curr. Pharm.* 2003.Des; 9: 2385–2402.
73. S.Park, T.L, Scheffler, D.E, Gerrard. Chronic high cytosolic calcium decreases AICAR-induced AMPK activity via calcium/calmodulin activated protein kinase II signaling cascade. *Cell Calcium.* 2011.
74. Sanders MJ, Grondin PO, Hegarty BD, Snowden MA, Carling D. Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. *Biochem J.* 2007; 403: 139–148.
75. Scalia R, Gong Y, Berzins B, Freund B, Feather D, Landesberg G, Mishra G. A novel role for calpain in the endothelial dysfunction induced by activation of angiotensin II type 1 receptor signaling. *Circ Res.* 2011 Apr 29; 108 (9):1102-11.

- 76.** Scalia R, Murohara T, Campbell B, Kaji A, Lefer AM. Lysophosphatidylcholine stimulates leukocyte rolling and adherence in rat mesenteric microvasculature. *The American journal of physiology.* 1997; 272:H2584-2590.
- 77.** Sessa WC. eNOS at a glance. *J Cell Sci.* 2004; 117: 2427–2429.
- 78.** Shah V, Wiest R, Garcia-Cardena G, Cadelina G, Groszmann RJ, Sessa WC. Hsp90 regulation of endothelial nitric oxide synthase contributes to vascular control in portal hypertension. *The American journal of physiology.* 1999; 277: G463-468.
- 79.** Shimabukuro M, Chinen I, Higa N, Takasu N, Yamakawa K, Ueda, S. Effects of dietary composition on postprandial endothelial function and adiponectin concentrations in healthy humans: a crossover controlled study. *Am J Clin Nutr.* 2007; 86 (4):923 – 8.
- 80.** Shrestha C, Ito T, Kawahara K, Shrestha B, Yamakuchi M, Hashiguchi T, Maruyama I. Saturated fatty acid palmitate induces extracellular release of histone H3: a possible mechanistic basis for high-fat diet-induced inflammation and thrombosis. *Biochem Biophys Res Commun.* 2013 Aug 9; 437(4):573-8.
- 81.** Stalker TJ, Gong Y, Scalia R. The calcium-dependent protease calpain causes endothelial dysfunction in type 2 diabetes. *Diabetes.* 2005; 54: 1132-1140.
- 82.** Stalker TJ, Skvarka CB, and Scalia R. A novel role for calpains in the endothelial dysfunction of hyperglycemia. *Faseb J.* 2003; 17: 1511-1513.
- 83.** Stremmel W, Pohl L, Ring A, Herrmann T. A new concept of cellular uptake and intracellular trafficking of long-chain fatty acids. *Lipids* 2001; 36(9):981 – 9.

84. Stuehr DJ. Mammalian nitric oxide synthases. *Biochim. Biophys. Acta* May 1999; 1411 (2–3): 217–30.
85. Sun C, Alkhoury K, et al. IRF-1 and miRNA126 modulate VCAM-1 expression in response to a high-fat meal. *Circ Res.* 2012 Sep 28;111(8):1054-64.
86. Suzuki K, Uchida K, Nakanishi N, Hattori Y. Cilostazol activates AMP-activated protein kinase and restores endothelial function in diabetes. *Am J Hypertens.* 2008; 21: 451–457.
87. Svedberg J, Bjorntorp P, Smith U, Lonroth P: Free-fatty acid inhibition of insulin binding, degradation, and action in isolated rat hepatocytes. *Diabetes.*1990; 39:570-574.
88. Takano M, Meneshian A, Sheikh E, Yamakawa Y, Wilkins KB, Hopkins EA, et al. Rapid upregulation of endothelial P-selectin expression via reactive oxygen species generation. *Am J Physiol Heart Circ Physiol.* 2002; 283 (5):H2054 – 61.
89. Talukdar S, Oh DY, Bandyopadhyay G, Li D, Xu J, McNelis J, et al. Neutrophils mediate insulin resistance in mice fed a high-fat diet through secreted elastase. *Nat Med.* 2012.
90. Tsubuki S, Saito Y, Tomioka M, Ito H, and Kawashima S. Differential inhibition of calpain and proteasome activities by peptidyl aldehydes of di-leucine and tri-leucine. *J Biochem.* 1996; 119: 572-576.
91. van Beijnum JR1, Rousch M, Castermans K, van der Linden E, Griffioen AW. Isolation of endothelial cells from fresh tissues. *Nat Protoc.* 2008; 3 (6):1085-91.
92. Vasanti S, Malik, Walter C, Willett and Frank B. Hu. Global obesity: trends, risk factors and policy implications. *Nat RevEndocrinol.* 2013 Jan; 9(1):13-27.

93. Vestweber D, Blanks JE. Mechanisms that regulate the function of the selectins and their ligands. *Physiol Rev.* 1999 Jan; 79 (1):181-213.
94. W.T. Cade, Diabetes-related microvascular and macrovascular diseases in the physical therapy setting, *Phys. Ther.* 2008; 88: 322–335.
95. Wang KK, Nath R, Posner A, Raser KJ, Yuen P et al. An alpha mercaptoacrylic acid derivative is a selective nonpeptide cell-permeable calpain inhibitor and is neuroprotective. *Proceedings of the National Academy of Sciences of the United States of America.* 1996; 93:6687-6692.
96. Wellen KE, Hotamisligil GS. Obesity-induced inflammatory changes in adipose tissue. *J Clin Invest* 2003; 112: 1785 – 1788.
97. Wong H, Yang D, Hill JS, Davis RC, Nikazy J, Schotz MC. A molecular biologybased approach to resolve the subunit orientation of lipoprotein lipase. *Proc Natl Acad Sci* 1997; 94: 5594–5598.
98. Wu Y, Song P, Xu J, Zhang M, Zou MH. Activation of protein phosphatase 2A by palmitate inhibits AMP-activated protein kinase. *J Biol Chem.* 2007; 282 (13):9777 – 88.
99. Zhang Y, Lee TS, Kolb EM, Sun K, Lu X, Sladek FM, Kassab GS, Garland T, Shyy JY. AMP-activated protein kinase is involved in endothelial NO synthase activation in response to shear stress. *Arterioscler Thromb Vasc Biol.* 2006; 26: 1281–1287.