

UC Riverside

UC Riverside Electronic Theses and Dissertations

Title

High Glucose Inhibits the AMPK-AKT2-ATF-2-MMP2 Pathway and Endothelial Cell Migration

Permalink

<https://escholarship.org/uc/item/49h8k28j>

Author

Smith, Lemar Irvin

Publication Date

2013

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA
RIVERSIDE

High Glucose Inhibits the AMPK-AKT2-ATF-2-MMP-2 Pathway
and Endothelial Cell Migration

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Biomedical Sciences

by

Lemar Irvin Smith

March 2013

Dissertation Committee:

Dr. John Y-J. Shyy, Chairperson

Dr. David A. Johnson

Dr. Victor Rodgers

Copyright by
Lemar Irvin Smith
2013

The Dissertation of Lemar Irvin Smith is approved:

Committee Chairperson

University of California, Riverside

Acknowledgements

I would like to acknowledge those individuals who have made such a lasting impact upon my training experience at the University of California. I would like to thank my research advisor Dr. John Y-J. Shyy for his guidance, support and encouragement to help me accomplish my research work here. I would also like to thank Ms. Traci Marin, Dr. Wei Sun, Dr. David A. Johnson and Dr. Victor Rodgers for their time, suggestions and valuable comments for my work and dissertation. I am thankful to all of my colleagues and friends for their generous help and precious suggestions.

ABSTRACT OF THE DISSERTATION

High Glucose Inhibits the AMPK-AKT2-ATF-2-MMP-2 pathway
and Endothelial Cell Migration
by

Lemar Irvin Smith

Doctor of Philosophy, Graduate Program in
Biomedical Sciences
University of California, Riverside, March 2013
Dr. John Y.-J. Shyy, Chairperson

There are numerous deleterious effects of repeated bouts of acute high glucose (HG) or chronic HG. At the molecular level, HG causes oxidative stress, increases advanced glycation end products which activate their cognate receptors (AGE/RAGE), and activate pro-inflammatory pathways. At the physiological level, tooth decay (lactic acid demineralization of enamel), gum disease, obesity (pro-inflammatory cytokines, adipokines), erectile dysfunction, vaginal infections result from HG. Important to this project, HG damages blood vessels leading to the cardiovascular complications in diabetes. HG accelerates macrovascular disease increase risk of strokes, myocardial infarction and renal failure. Further, HG promotes an increased susceptibility to dermal breakdown, forming a portal of entry for microbial infections. This is accompanied by inadequate wound healing of decubitus ulcers, trauma/accident-induced injury or cutaneous infections.

An integral component of wound healing is the process of neovascularization or reperfusion of the injured or ischemic area to supply necessary nutrients and for removal of necrotic or damaged cells and debris. A primary component of neovascularization is endothelial cell (EC) migration to sites of injury. It is well established that HG conditions result in disturbed endothelial migration and wound healing. My specific aim is to delineate the molecular mechanisms of the HG impaired EC migration. The working hypothesis of this project is that high glucose inhibits the pro-angiogenic AMP-activated kinase, thereby inhibiting matrix metalloproteinase-2 collagen cleavage and matrix degradation, which under physiological glucose levels, facilitates wound healing through neovascularization. This pathway is mediated by the phosphorylation and activation of AKT2 (protein kinase B- β), which subsequently increases activating transcription factor 2 (ATF-2) binding to the MMP-2 promoter to increase its expression and activity.

Table of Contents

Acknowledgments.	iv
Abstract.	v
Table of Contents.	vii
List of Figures.	viii
Abbreviations.	ix
1.0 Chapter 1: Background.	1
2.0 Chapter 2: High Glucose inhibits the AMPK-AKT2-ATF-2-MMP2 pathway and EC Migration.	11
2.0 Introduction.	12
2.1 Methods.	15
2.2 Results.	19
2.3 Discussion.	23
2.4 Figures	27
3.0 Chapter 3: Perspectives and Conclusions.	35
4.0 References.	37

List of Figures

1. EC migration under LG and HG with and without AICAR.	27
2. Immunoblot of EC under LG vs. HG for AMPK.	28
3. AKT2 peptide assay and AKT2 full length autoradiography.	29
4. MMP-2 activity assay.	30
5. In-vitro wound and EC migration assay.	31
6. ChIP assay for ATF-2 and bound MMP-2 promoter amplification.	32
7. MMP-2 expression analysis of mRNA and protein via qPCR and immunoblot.	33
8. AMPK-AKT2-ATF-2-MMP-2 Pathway.	34

Abbreviations

AGE : advanced glycation end product

AICAR : 5-aminoimidazole-4-carboxymide-1- β -d-ribofuranoside

AKT2 : protein kinase B2

AMP : adenosine monophosphate

AMPK : ATP-activated protein kinase

AP-1: activating protein-1

ATF-2 : activating transcription factor-2

ATP : adenosine triphosphate

ChIP : chromatin immunoprecipitation

DNA : deoxyribonucleic acid

EC : endothelial cell

ECL : Emerson chemiluminescence substrate

ECM : extracellular matrix

eNOS : endothelial nitric oxide synthase

EPC : endothelial progenitor cell

FGF : fibroblast growth factor

GSH: glutathione

HG : high glucose

HIF-1 α : hypoxia-inducible factor-1 α -dependent

ILK: integrin-linked kinase

LG : low glucose

MMP-2 : matrix metalloproteinase-2

MnSOD : manganese superoxide dismutase

mRNA : messenger ribonucleic acid

mTOR : mammalian target of rapamycin

NAD⁺ : nicotinamide adenine dinucleotide

NADPH : nicotinamide adenine dinucleotide phosphate

NFκB : nuclear factor kappa B

NO : nitric oxide

PGC-1α : peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PI3K : phosphatidylinositide 3-kinase

PKB: protein kinase B

PKC : protein kinase C

qPCR: quantitative polymerase chain reaction

RAGE: receptor for advanced glycation end products

ROS : reactive oxygen species

SDH: sorbitol dehydrogenase

siRNA : silencing RNA

TGF-β: transforming growth factor beta

VEGF : vascular endothelial growth factor

Chapter 1

1.0 Background

Hyperglycemia (HG) is broadly defined as “high blood sugar”. More precisely, elevated levels of D-glucose. Glucose levels vary before and after meals, and at various times of day. The normal range for most people after fasting for at least 8 hours is 80 to 110 mg/dL (4 to 6 mM). Postprandial or after-meal hyperglycemia is defined as a blood sugar usually greater than 180 mg/dL (10 mM). In people without diabetes postprandial glucose rarely exceeds 140 mg/dL (7.7 mM). However, occasionally after a large meal, a 1-2 hour post-meal sugar level can reach 180 mg/dL. A subject with a consistent fasting glucose level between 100 and 126 (American Diabetes Association Guidelines¹) is considered hyperglycemic, while above 126 mg/dL or 7 mM is generally held to have diabetes. Chronic levels exceeding 7 mM can produce organ damage.¹

There are a multitude of causes or risk factors of HG. Dietary excess of carbohydrates (especially those with high glycemic index or glycemic load) often result in acute and transient HG, excess weight gain and obesity. Diabetes due to insufficient insulin (type 1) or insulin resistance (type 2) is by far the most common cause of chronic HG and is the defining characteristic of the disease. Other risk factors or causes of HG include, but not limited to: 1) critical illness (eg, trauma, burns, infection, surgery), 2) endocrine dysfunction (eg, thyroid, adrenal, pituitary, adiponectin), 3) medications (eg, corticosteroids, beta blockers,

epinephrine, thiazide diuretics, niacin, protease inhibitors, amphetamine, some antipsychotic agents and skipping or forgetting insulin or oral glucose-lowering medicine), 4) psychological stress “fight-or-flight” response with release of cortisol, epinephrine and aldosterone, 5) environmental stress (eg, noise, air pollution, crowding, traffic congestion, terrorism, natural disasters and extremes of temperature), 6) advanced age, 7) genetics, eg, 50 % of adult Pima Indians are diabetic.⁸⁵

Several reasons highlight the importance of HG to public health. The causes of HG are highly prevalent. Twenty-six million people in the USA are diabetic and 79 million are pre-diabetic, therefore, over 100 million are hyperglycemic (2011 National Diabetes Fact Sheet). An estimated 250 million people world wide are diabetic and 1 billion pre-diabetic, therefore, 1.2 billion are hyperglycemic.⁸⁷ The incidence of obesity (childhood, adolescent and adult) and insulin resistance are increasing.⁸⁸ The average life expectancy especially in developing countries is increasing and advanced age is a HG risk factor. Chronic or repeated episodes of acute HG causes serious cardiovascular complications. The cost of medical care for HG related conditions is very high (\$174 billion for total costs of diagnosed diabetes in the USA in 2007, including \$116 billion for direct medical costs, and \$58 billion for indirect costs, eg disability, work loss and premature mortality.⁸⁹

The correlation between chronic and post-prandial hyperglycemia and risk for cardiovascular disease is supported by many epidemiological studies.²⁻⁷ In

fact, a post prandial (1hr) glucose level of type 2 diabetics is a stronger predictor of myocardial infarction as demonstrated in The Diabetes Intervention Study.⁸ The vascular complications of persistent HG affect both the (macrovasculature) larger vessels and microvasculature (arterioles, capillary bed, venules) Aberrant angiogenesis is the gateway to diabetic complications. A perplexing feature of HG modulated angiogenesis, is the tissue specificity. In the same individual, HG can simultaneously cause increased angiogenesis in some tissues, eg, increased tumor angiogenesis, diabetic retinopathy, age related macular degeneration, aortic plaque atherogenesis, while decreasing angiogenesis in other tissues (coronary collaterals, ischemia reperfusion, cutaneous wound healing). Obviously for new blood vessels to form from preexisting vessels (ie, angiogenesis) individual cell types, specifically ECs, which compose small vessels have to move from where they are to where they are needed. ECs must migrate for angiogenesis to occur and HG impairs EC migration in superficial wound repair.⁹⁰

Wound healing occurs as four overlapping phases: 1) hemostasis as a result of platelet aggregation and clotting cascades, 2) inflammatory response initiating phagocytosis and recruitment of factors important for healing and proliferation, 3) proliferation including new tissue formation and angiogenesis and 4) remodeling of tissue and vessel maturation.²⁶ Vessel maturation includes angiogenesis also called neovascularization. Angiogenesis occurs concomitantly with fibroblast proliferation and is in part due to endothelial cells migrating to the

site of insult and is necessary for wound healing as it provides nutrients and oxygen to cells involved in healing such as fibroblasts and epithelial cells.²⁷⁻²⁸

Angiogenesis is the generation of new capillaries from pre-existing capillaries and is essential for embryonic development, tissue remodeling, response to hypoxemia, wound healing and much more.²⁷⁻²⁸ This process is triggered by pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). Angiogenesis is a systemic process. It involves vascular leakage, hemostasis in tissues, activation of elements of the immune system, recruitment of bone-marrow-derived progenitor cells, activation of proteases, and recruitment and proliferation of perivascular cells during vascular maturation. A focus of this study is the underlying molecular mechanisms responsible for reduced endothelial cell (EC) migration under HG conditions. Endothelial cells travel to the injured site via pseudopodia allowing them to migrate to and through extracellular matrices.²⁹ Fibronectin, a component of a fibrin clot, chemotactically recruits ECs to the site of the insult.²⁹ Both hypoxia and lactic acid build up also initiate angiogenic factor production.²⁹

Although there are many components to signaling cascades that initiate EC migration, one important group of molecules includes metalloproteinases (MMPs), which degrade basement membranes and the extracellular matrix (ECM) by collagen cleavage to allow cell migration, proliferation and angiogenesis.³⁰ Once tissue is adequately perfused due to neovascularization,

angiogenic chemotactic recruitment subsides because the cells producing these chemicals are no longer hypoxic or in a high lactic acid environment.²⁹

Complications due to impaired neovascularization is the leading cause of morbidity/mortality in diabetes mellitus patients.³¹ One co-morbidity of chronic hyperglycemia is impaired wound healing largely because of ineffective angiogenesis.³²⁻³³ A myriad of mechanisms are responsible for the pathological consequences of hyperglycemia. For example, hyperglycemia-induced high ROS production ultimately results in poor peripheral perfusion, decreased growth factor response, EC dysfunction, and aberrant inflammatory response.³⁴ Underlying the above mentioned is inadequate NO bioavailability. Impaired NO production is a hallmark of endothelial dysfunction and is necessary for growth factor expression and neovascularization.³⁵⁻³⁶ Chronic hyperglycemia is often accompanied by insulin resistance and hence impaired insulin signaling. Insulin is important for normal NO bioavailability through activation of the PI3K (phosphatidylinositide 3-kinase)-Akt [Protein kinase B (PKB)] pathway leading to eNOS phosphorylation and activity. Importantly, the PI3K-Akt pathway has been shown to be down regulated in the aortas of diabetic animal models and diabetic human patients.³⁷ Another consequence of decreased NO bioavailability is reduced circulating endothelial progenitor cells (EPCs) and impaired bone marrow EPC function. EPC are necessary for angiogenesis as they provide an additional source of proliferation, migration and differentiation of EC.³⁸⁻³⁹

Angiogenesis is dependent upon vascular remodeling or collateral growth and requires many physiological and other molecular factors. Shear stress and hypoxia are physiological stimuli that accelerate angiogenesis.⁴⁰⁻⁴¹ Additionally, integrin, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and transforming growth factor beta (TGF- β) are important molecules for vessel formation and are dependent upon physiological NO levels.⁴²⁻⁴⁴ However, aberrant ROS production initiates alterations in pathways involving these molecules as well as those of inflammatory mediators, such as NF-kB, and AGEs, all of which reduce vascular growth and stabilization.^{32, 45-49}

A major reason for this correlation is the exaggerated production of superoxide or reactive oxygen species (ROS) by mitochondrial electron transport chain upon high glucose load.⁹ Elevated ROS level initiates pathways involved in inflammation and other events that ultimately lead to endothelial dysfunction and vascular disease.⁹⁻¹⁰ For example, high levels of superoxide will react with nitric oxide (NO) to produce peroxynitrite and nitrotyrosine that are key to microvascular damage.¹¹⁻¹³ However, hyperglycemia and increased ROS formation also inhibits endothelial nitric oxide synthase (eNOS) activity. eNOS activity and NO bioavailability are biomarkers of endothelial health and function.¹⁴ Examples of pathways shown to be involved in ROS initiated vascular damage include: 1) polyol pathway flux, 2) increased formation of AGEs (advanced glycation end products) and increased expression of the AGE receptors, and 3)

activation of protein kinase C (PKC) isoforms. The polyol pathway involves the conversion of glucose to sorbitol by aldose reductase followed by sorbitol oxidation to fructose by sorbitol dehydrogenase (SDH), which uses NAD⁺ as a cofactor. In the presence of hyperglycemia, this results in a depletion of NADPH. NADPH is a cofactor necessary to regenerate reduced glutathione (GSH), which is a potent ROS scavenger. Over activation of this pathway leads to increased ROS production and vascular pathology.¹⁵ ROS production is also stimulated by both intracellular and extracellular AGE. AGE are a heterogeneous group of molecules formed from the nonenzymatic reaction of reducing sugars with free amino groups of proteins, lipids, and nucleic acid.¹⁶⁻¹⁷ This is in part due to abnormal interaction of extracellular AGE-modified molecules with integrins. However, the mechanisms of AGE-induced vascular pathology cover a wide spectrum including activation of the pro-inflammatory transcription factor nuclear factor (NF- κ B) pathway.¹⁸⁻¹⁹ The over activation of the β and δ isoforms of protein kinase C (PKC) is primarily due to hyperglycemia, specifically, of PKC in vascular cells.²⁰⁻²² Over active PKC leads to increased ROS and decreased eNOS function through its downstream pathways.²³

Lastly, wound healing occurs as a cellular response to injury and involves activation of keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets. Collateral vessel formation is highly dependent upon monocytes/macrophage function, particularly through VEGF. Normal monocyte

function is impaired in diabetic patients and diabetic animal models.^{32-33,43} Recall that hyperglycemia increases AGEs, which has been shown to alter VEGF signaling to inhibit endothelial growth and migration, monocyte, and EPC recruitment and release from bone marrow.⁵⁰ Additionally, diabetic patients present with reduced chemotaxis in polymorphonuclear neutrophils (PMNs).⁵¹

AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase known to regulate energy homeostasis, reduce oxidative stress, and improve mitochondrial function. AMPK's anti-oxidative effects are also true in the presence of hyperglycemia as AMPK activation enhances MnSOD (superoxide dismutase) activity in ECs⁵², and acts as a master regulator of eNOS through phosphorylation.⁵³ However, it is also a positive regulator of endothelial cell migration and differentiation in the presences of hypoxia to facilitate angiogenesis.⁵⁴ One mechanism by which AMPK initiates angiogenesis is through VEGF production independent of eNOS activity.⁵⁵⁻⁵⁶ Additionally, EPC differentiation, and angiogenesis *in vitro* and *in vivo* is promoted by AMPK⁵⁷, even in diabetic patients by suppressing EPC mitochondrial superoxide level to rescue their angiogenic function through MnSOD induction.⁵⁸

Aside from promoting angiogenesis, AMPK increases intracellular calcium levels in microvascular endothelial cells thereby initiating signaling events leading to restoration of cell-cell adhesions after barrier disruption *in vitro* and in the intact lung *in vivo*.⁵⁹

Importantly, elevated ROS activates AMPK possibly by reducing ATP levels. Further, 5-aminoimidazole-4-carboxamide-1- β -*D*-ribofuranoside (AICAR) mimics AMP, which binds to the gamma subunit of AMPK to expose its active site for phosphorylation and increased activity. Metformin is also a known AMPK activator and is used to improve insulin sensitivity in diabetics. ROS, to a certain threshold, AICAR and metformin increase AMPK phosphorylation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) and increases its promoter activity to initiate mitochondrial biogenesis.⁶⁰ Accumulating evidence indicate mitochondrial ROS functions as signaling molecules in vascular endothelial cells. Mitochondrial ROS signaling has been implicated in the regulation of vascular tone, adaptive changes to mechanical stimuli, and vascular responses to hypoxia-reoxygenation. However, excess production of mitochondrial ROS leads to a disruption of normal ROS signaling and mitochondrial dysfunction, which contributes to the pathogenesis of cardiovascular disease.

Mammalian cells express three different AKT isoforms (AKT1, 2, and 3; also known as PKB α , β , and γ ,) all of which are encoded by distinct genes. Although there is some overlap, they all have distinct cellular roles.⁶¹ AKT2 (PKB β), plays an important role in EC migration, proliferation, differentiation, apoptosis, and metabolism. Notably, AKT2 gene disruption results in growth deficiency, impaired insulin signaling, and predisposes to insulin resistance and diabetes.⁶⁰⁻⁶² As previously discussed, in ECs, growth factor-induced responses

are largely mediated by the activation of the PI3 kinase-AKT1 signaling cascade.⁶⁴ The VEGF-PI3Kinase-AKT signaling cascades are believed to play the most significant role in angiogenesis, cell survival, and cell migration.⁶⁴⁻⁶⁶ This pathway is also vital for wound repair, but it seems AKT2 may be more important for this physiological response than AKT1. For example, double AKT1/AKT2 null mice have severely impaired skin regeneration and development than AKT1^{-/-} mice. A deficiency of two AKT isoforms leads to a translucent skin phenotype and the individual skin layers are much thinner than in wild type mice.⁶⁷ AKT2 is specifically necessary for wound-induced cell migration and chemotaxis. The interaction between integrin-linked kinase (ILK) and AKT2 is required MMP-2 synthesis and EC migration.⁶⁸ One study shows that ILK–AKT2-induced MMP-2 expression depends on phosphorylation and activation of AKT2 to facilitate matrix degradation.⁶⁸

Interestingly, hypoxia enhances EC migration through matrix metalloproteinase 2 (MMP-2).⁶⁹ This effect does not seem to be dependent upon tube formation but rather the facilitation of cell migration via collagen IV degradation and autocrine signaling to determine cell fate possibly through $\alpha_v\beta_3$ and paxillin signaling dependent upon MMP-2.⁶⁹⁻⁷¹ The hypoxia-induced production of MMP-2 appears to involve both transcriptional and posttranscriptional regulation.⁶⁹ Although MMP-2 is considered a housekeeping molecule, small changes in its level have important physiological.⁷²⁻⁷³ MMP-2 expression has been shown to be mediated by activating protein-1 (AP-1),

hypoxia-inducible factor-1 α (HIF-1 α), as well as activating transcription factor 2 (ATF-2).⁷⁴⁻⁷⁶

In conclusion, hyperglycemia produces a state of oxidative stress through the over projection of mitochondrial ROS, decreased eNOS function and NO bioavailability, and inflammation. Chronic oxidative stress decreases EC migration, neovasularization and impaired tissue regeneration and wound healing.

Chapter 2

2.0 Introduction

All causes of chronic or repeated hyperglycemia (HG) are associated with an increased risk of cardiovascular complications. Our subject area of interest is the vascular ramifications of sustained HG. Prior to these experimental studies, it is well documented that subjects with HG, for example diabetics, have an increased incidence and severity of cardiovascular complications affecting both the macrovasculature and microvasculature. Microvascular complications include decreased wound healing and neovascularization after injury. Neovascularization can be categorized into arteriogenesis and angiogenesis. Angiogenesis is the sprouting of new capillaries from pre-existing vessels. Capillaries are composed primarily of a single monolayer of ECs surrounded by a thin basement membrane. EC motility or migration is an integral component of angiogenesis, and is impaired under HG conditions resulting in chronic refractory non-healing wounds. Healing and repair of wounds (eg ankle/foot pressure ulcers, decubitus ulcers, surgical incisions, tooth extractions, burns or trauma) is retarded in the HG tissue environment. A major contributing pathophysiological process responsible for the HG impaired wound healing is reduced revascularization and angiogenesis. EC migration is a requirement of angiogenesis and is impaired under HG conditions. The molecular basis for the HG impaired EC migration is not entirely understood. The purpose of our research project was to test the

hypothesis that HG impairs EC migration by inhibiting signaling through the AMPK/AKT2/ATF-2/MMP-2 cascade.

It is well documented that AMPK and SIRT1 are energy sensing enzymes systems or metabolic “fuel gauges”. Supraphysiological (HG) levels of glucose is synonymous with a cellular high energy charge and inhibition of AMPK. The rationale for the experimental design was to perform a bioinformatic proteome wide screen for potential AMPK target substrates that have known functionality in EC migration. AKT, also known as Protein Kinase B (PKB), is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as glucose metabolism and cell migration. AKT isoforms ($\alpha/1$, $\beta/2$, $\gamma/3$) are responsive to several growth factors for example insulin and vascular endothelial growth factor (VEGF). The three AKT isoforms have both overlapping and distinct functions. The AKT2 isoform was not previously known to be a direct AMPK target. AKT2 is known to be involved in various aspects of cell motility, migration and metastasis. Interestingly, we found that AKT2 has an amino acid sequence that closely matches the AMPK consensus binding sequence. Additional experimental approaches utilized specific pharmacological activators and genetic inhibitors of key signaling molecules involved in MMP-2 expression, activity and cell migration under LG and HG conditions. We tested the hypothesis that under HG, AMPK phosphorylation and activity is attenuated. Additionally we propose that AKT2 is a direct AMPK target and that activated AKT2 increases MMP-2 expression and activity through ATF-2 binding. Therefore we propose

that HG contributes to decreased EC migration by reduced AMPK/AKT2/ATF-2/MMP-2 signaling.

Cell migration is a coordinated response between intracellular and extracellular events. Degradation of extracellular matrix (ECM) components by EC secreted proteases is requisite for EC migration. In the capillary vasculature the primary ECM is collagen in the basement membrane. Matrix metalloproteinases (MMP-2) degrade type IV collagen. Activating transcription factor- 2 (ATF-2) is known to bind to the MMP-2 promoter and is required for MMP-2 expression and activity (Ref). We thus proposed the hypothesis that HG impaired AMPK/AKT2 activation of ATF-2 and its subsequent binding to the MMP2 promoter. Additionally we tested whether the reduced ATF-2 binding effected MMP-2 expression and activity.

The importance of gaining increased insight and understanding of the molecular basis of HG impaired EC migration is highlighted by the increasing prevalence of diabetes (DMII) and prediabetes in the USA and worldwide. In addition, all other causes or associated risk factors for HG share the deleterious vascular complications causing increased morbidity and mortality in the affected populations. Consequently, delineation of a contributing molecular pathway in HG suppressed EC migration, may provide more focused targets for therapeutic intervention. This study makes a modest contribution to the field of prevention, management and treatment of difficult chronic wound healing, eg, foot ulcers in the population with persistently elevated plasma glucose levels.

2.1 Methods

Cell Migration (Scratch Assay)

ECs were plated in six well plates and allowed to grow to confluency. A sterile 1 ml pipette tip was used to create a linear “scratch” i.e. an acellular zone. Cell migration was quantified by enumerating the cells crossing into the acellular wounded area over a 16 to 24 h interval.

Immunoblotting

Following treatment, cells were lysed in 10 mM Tris (pH 7.4), 0.1 M NaCl, 1 mM EDTA, 1 mM EGTA, 1mM NaF, 20 mM $\text{Na}_4\text{P}_2\text{O}_7$, 2 mM sodium orthovanadate (Na_3VO_4), 0.1% SDS, 0.5% Sodium Deoxycholate, 1% Triton X-100, 10% Glycerol, 1 mM PMSF and protease inhibitors. Cell lysates were resolved via 8% SDS-PAGE, blocked with 5% milk, rinsed with TBST, probed with primary rabbit antibodies (1:1000) to p-AMPK, t-AMPK and β -actin. Membranes were incubated in goat anti-rabbit secondary antibody (1:2000) for 1h at room temperature. Signal was generated by Emerson chemiluminescence substrate (ECL) and image captured on HyBlot CL autoradiography film.

Kinase assay

AKT2 peptide assay reaction mixture was composed of 50 mM HEPES (pH 7.4), 25 mM MgCl_2 , 1 mM AMP, 1 mM ATP, 3 mM peptide, [^{32}P] γ ATP and

1 pM AMPK. SAMS peptide was used as positive control and no peptide as negative control. AMPK kinase assays utilizing full-length AKT2 substrate were conducted in 50 mM HEPES (pH 7.4), 0.375 mM AMP, 0.375 mM ATP, 9 mM MgCl₂, 11 pM AMPK and 2 micrograms of recombinant AKT2 protein in a 50 µl reaction volume at 37 °C for 1 h. Proteins were then resolved using SDS-PAGE, stained with Coomassie blue, and submitted to autoradiographic analysis. Peptide reactions were terminated by blotting samples onto Whatman filter paper (P81 cat# 3698-325) and rinsed three times in 1% phosphoric acid. Individual filter papers were then washed in acetone and allowed to air dry for 5 min. Filter papers were placed into separate scintillation vials and 1 ml scintillation fluid was added prior to measurements with a Beckman LS 6500 scintillation counter.

Genomic DNA isolation

Cells were lysed in 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₄P₂O₇, 2 mM Na₃VO₄, 0.1% SDS, 0.5% Sodium Deoxycholate, 1% Triton X-100, 10% Glycerol, 1 mM PMSF, and protease inhibitors. Cell lysates were extracted with phenol:chloroform (1:1) and the RNA was removed with the addition of RNase A suspended in 20 mM Tris, pH 7.4. After 1 h incubation at 37 °C, reactions were precipitated with an equal volume of isopropanol and a 1:10 volume of potassium acetate, and re-suspended in 50 µl nuclease free H₂O.

ChIP assay

Following cell treatment, protein was cross linked to DNA with the addition of formaldehyde. Cells were washed three times with phosphate buffer saline (PBS) and then scraped from the surface of the plates in 500 μ l FA lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS and Halt Protease inhibitors). The resulting cell lysates were sonicated with a Bioruptor 200. ATF-2 was immunoprecipitated overnight at 4 °C with protein A conjugated sepharose beads in the presence of desired antibody. The beads were washed three times with wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.0, 150 mM NaCl, 20 mM Tris-HCl (pH 8.0) and eluted in 100 μ l elution buffer (1% SDS, 100 mM NaHCO₃). All DNA samples were purified using Qiagen PCR purification kit prior to qPCR analysis. The following primers were used for qPCR analysis of immunoprecipitated DNA: GCAGAAGGAAAGAGGTAAGGAAG and GAAGGAATGGTCAGAAACAGATG.

mRNA Quantification

RNA was purified using TRIzol reagent from Life technologies. Two μ g of RNA was converted to cDNA using Promega reverse transcriptase according to the manufacturer's instructions. cDNA was then quantified via qPCR using cyber

green qPCR master mix purchased from Bio-Rad. Results were calculated using the delta-delta ct method. The following primers were used for qPCR analysis:

1105F TTGAGAAGGATGGCAAGTACG and
1596R TGGTGTAGGTGTAAATGGGTG.

MMP-2 Assay

Endothelial cells were transfected with either Ctrl or AKT2 siRNA and treated with either AMPK activators or inhibitors. AICAR (1 mM) activates AMPK and Compound C (20 μ M) inhibits AMPK respectively. Colorometric results read at 405 nm with a microplate spectrophotometer per protocol provided from Amersham Matrix Metalloproteinase-2 (MMP-2) Biotrak Activity Assay System RPN2631 protocol.

Statistical Analysis

Data are expressed as means \pm SEM of at least three independent experiments. Comparisons of mean values between two groups were evaluated using a two-tailed Student's t test, Wilcoxon signed-rank test or Mann-Whitney U test. Unless otherwise indicated, * p <0.05 was considered statistically significant.

2.2 Results

High glucose inhibits EC migration and is reversed with AMPK activation.

To determine if pathophysiological glucose levels negatively affect EC migration and can be rescued upon AMPK activation, ECs were grown in LG (5.6 mM) or HG (25 mM) D-glucose medium and allowed to grow to confluency in 6 well plates. Once confluent, the bottom of the wells were scratched and photomicrographs were taken at time 0 and 16 h, with and without AICAR (1 mM) treatment. As illustrated in Fig. 1, HG decreased cell migration and AICAR reversed the suppressive effect of HG.

High glucose decreases AMPK phosphorylation.

To further demonstrate AMPK's role in high glucose inhibition of cell migration, ECs were grown in LG and HG and the protein levels of p-AMPK (T172) and t-AMPK measured. As illustrated in Fig. 2, phosphorylation of AMPK at T172 was decreased when cells are grown in HG for 24 h. The p-AMPK/t-AMPK under HG is reduced by ~55 % compared to LG. This confirms AMPK's role as an energy sensor and its inactivation upon energy excess.

AMPK phosphorylates AKT2.

AKT2 is known to be involved in EC migration. Since HG is known to inhibit AMPK and EC migration, we wanted to determine if AMPK could directly phosphorylate AKT2. Two AKT2 serines (residues S242 and S268) are located

within strings of residues that are consistent with the consensus sequence for AMPK substrate targets (Fig. 3C). Consequently, we examined the ability of AMPK to phosphorylate peptides composed of residues in these strings (Fig. 3D). Fig. 3A illustrates the results of this experiment. AKT2 peptides containing either S242 or S268 were phosphorylated to an extent comparable to the SAMS peptide (a standard positive control substrate for AMPK kinase activity). Conversely, peptides containing either S242A or S268A were significantly less phosphorylated, with levels comparable to the blank no-peptide negative control (Fig. 3A). Further, using full-length AKT2 substrate and stringent conditions, AMPK-dependent [³²P] γ-phosphate incorporation was observed (Fig. 3B).

AMPK activation increases MMP-2 activity through AKT2.

To determine if both AMPK and AKT2 play a role in increasing MMP-2 cleavage of collagen, ECs were transfected with either AMPK siRNA , AKT2 siRNA or control(Ctrl) siRNA, and treated with AMPK activators (AICAR, 1mM) or inhibitors (Compound C, 20 μM/data not shown). The results of these experiments are illustrated in Fig. 4A. AICAR activation of AMPK increased MMP-2 activity greater than two fold over basal levels but not in the AMPK siRNA or AKT2 siRNA transfected cells. This evidence suggests that AMPK activity is positively correlated with MMP-2 activity through AKT2. AMPK inhibition with Compound C caused no significant change in MMP-2 activity (data not shown). This assay was repeated in LG and HG medium. MMP-2 activity under LG

culture conditions mimic AMPK activation with AICAR under HG conditions. MMP-2 activity under HG conditions is comparable to that of ECs cultivated under LG and transfected with AMPK siRNA or AKT2 siRNA (Fig. 4B).

HG decreased EC migration is AMPK/AKT2/ATF-2/MMP-2 dependent.

It has been previously reported that ATF-2 binds to the MMP-2 promoter to increase its transcription. Additionally we have provided evidence that AMPK activation increased MMP-2 activity, while HG decreased MMP-2 activity. We hypothesized that AMPK and AKT2 are upstream signaling molecules mediating ATF-2 binding and MMP-2 activity. To elucidate the physiological relevance of LG versus HG and the dependence on the proposed signaling pathway in the context of EC migration, we performed a set of migration experiments on ECs transfected with siRNA (AMPK, AKT2, or ATF-2) and grown in LG or HG. HG caused a two fold reduction in EC migration compared to LG transfected with control siRNA (Fig. 5). EC migration was attenuated under both LG and HG when transfected with AMPK siRNA. EC migration was dramatically diminished under LG and HG when transfected with either AKT2 siRNA or ATF-2 siRNA (Fig. 5).

AMPK and AKT2 are required for ATF-2 binding to the MMP-2 promoter and increased protein expression.

To determine the physiological role of AMPK and AKT2 in ATF-2 binding to the MMP-2 promoter, we genetically knocked down AMPK or AKT-2 expression

with siRNA specific to these two enzymes. Utilization of chromatin immunoprecipitation (ChIP) to pull down ATF-2 and quantitative polymerase chain reaction (qPCR) to amplify any bound chromatin, we demonstrated a greater than two fold increase in bound ATF-2 to the MMP-2 promoter in the AMPK stimulated / control transfected ECs (Fig. 6A). HG / control transfected ECs demonstrated a reduction of approximately half of the ATF-2 binding to the MMP-2 promoter compared to LG (Fig. 6B). The evidence indicates that both AMPK and AKT2 are necessary for ATF-2 binding to the MMP-2 promoter under low energy conditions.

AMPK and AKT2 role in MMP-2 expression and HG attenuated MMP-2 expression

To determine if ATF-2 binding to the promoter of MMP-2 under AMPK activating conditions increase MMP-2 expression, ECs were transfected with AMPK, AKT2, ATF-2 or control siRNA and treated with AICAR or grown in LG or HG and the MMP-2 protein and mRNA levels were measured. Both MMP-2 mRNA and protein levels increased with AICAR treatment in cells transfected with Ctrl RNA, but not in cells transfected with AMPK siRNA or AKT2 siRNA or in HG conditions (Fig 7A-C).

2.3 Discussion

The Center for Disease Control and World Health Organization estimate that more than 100 million people in the USA and more than 1.2 billion people world wide are currently diabetic or prediabetic. The diabetic incidence is expected to increase dramatically over the next decade, forecasting a pending epidemic (Ref). Given that the defining symptom of diabetes is hyperglycemia and that the principal cause of mortality in diabetics are cardiovascular complications it is imperative to have a clear understanding of the molecular basis of HG impairment of vascular function. This project aims to gain additional insight into the signaling pathways mediating HG impairment of EC migration in the context of vascular dysfunction. The major finding of this project is that diminished signaling through the AMPK/AKT2/ATF-2/MMP-2 pathway contributes to HG impairment of EC migration. A bioinformatic genome wide screen of potential AMPK targets suggested AKT2 as a putative AMPK substrate. Given that AMPK plays a pivotal role in cellular energy homeostasis and that AKT2 modulates EC migration and is a substrate for stress related kinases (Ref), we used pharmacological and genetic approaches to investigate the role of AMPK/AKT2/ATF-2 in HG impaired EC migration.

We utilized an endothelial cell monolayer to model wound healing where a narrow path of cells is scraped off and a time-course of cell migration into the scraped area is monitored. Hyperglycemia significantly affected migration

responses of ECs at the cellular edge. The effect of hyperglycemia on cell locomotion differed from the action of mannitol, another hyperosmolar agent used as an osmotic control in the low glucose medium. Clearly, the major limitation of this work is that it is an in vitro study and it does not examine other physiological relevant parameters that could impact EC, eg, oxygen tension, vascular growth and inhibitory factors, mechanical intercellular and cell-to-extracellular matrix traction forces, blood and plasma constituents, and other cell types. For sure, the aforementioned limitations are also a strength in that it allows for less confounding and more control of a single variable, ie, D-glucose concentration.

It is well established that HG affects migratory capacity of many cell types relevant to superficial wound healing in addition to ECs, eg, keratinocytes, bone marrow derived endothelial progenitor cells (EPC), fibroblast, pericytes, leukocytes (monocytes and neutrophils) and vascular smooth cells (VSMC). Our results validate the HG inhibitory effect on EC migration. However our main objective is to investigate the molecular basis for HG mediated impairment of EC migration, ie, AMPK/AKT2/ATF-2/MMP-2 signaling, in the context of angiogenesis.

It is accepted as conventional truth that AMPK is sensitive to cellular “energy charge” and is deactivated under HG conditions. It is also established that AKT2 is one of three AKT isoforms that has increased specificity for glucose

homeostasis, eg, AKT2^{-/-} mice are insulin resistant and hyperglycemic. It is known that ATF-2 binding to the MMP-2 promoter increases its expression and activity, ie, ECM/collagen degradation. Our study provides a modest novel contribution to the mechanistic basis linking cellular energy status to EC migration in an in-vitro wound healing model system. We show for the first time that HG contributes to reduced EC migration via AMPK/AKT2/ATF-2/MMP-2 dependent pathway.

These observations may be relevant to the pathogenesis and management of accelerated atherosclerosis and microvascular disease associated with the chronic or recurrent acute hyperglycemia.⁸⁰ Endothelial cell migration is essential to angiogenesis. Angiogenesis is essential for wound healing, tissue repair and is often impaired in the diabetic or hyperglycemic state. This molecular pathway may offer a more precise target for therapeutic intervention in subjects with poor wound healing, eg, topical MMP-2 preparations. However, it is generally easier to make safe and effective inhibitors rather than activators. The logical implications of this project point to the utility of addressing the root cause of decreased wound healing in HG. Minimize HG conditions.

There are many questions raised by this project. Given that a excess nutrient load, ie, HG decrease EC migration, does moderate caloric restriction (LLG) cause posttranslational changes, eg, SIRT1 catalyzed deacetylation of AKT2, resulting in increased EC migration? Is there cross-talk between AMPK

and SIRT1 regulation of AKT2/ATF2/MMP-2 signaling, eg, additive or synergistic effects? Can the HG inhibitory effect on EC migration be mitigated by increased antioxidants? How do concomitant changes in Insulin signaling affect AKT2/ATF-2/MMP-2 axis and EC migration? Why is hyperbaric oxygen therapy promoted as an effective treatment for diabetic foot wounds, when hypoxia activates AMPK?

Next steps in the project should include: (1) Mutating the full length AKT2 protein at the proposed phosphorylation sites and monitoring the AMPK activity, (2) Use of ATF-2 phosphospecific antibody and (3) Screening of commonly used nutraceutical and pharmaceuticals (eg, statins, metformin, resveratrol, ACE inhibitors) for pleiotropic effects on EC migration signaling through AMPK/AKT-2.

2.4 Figures

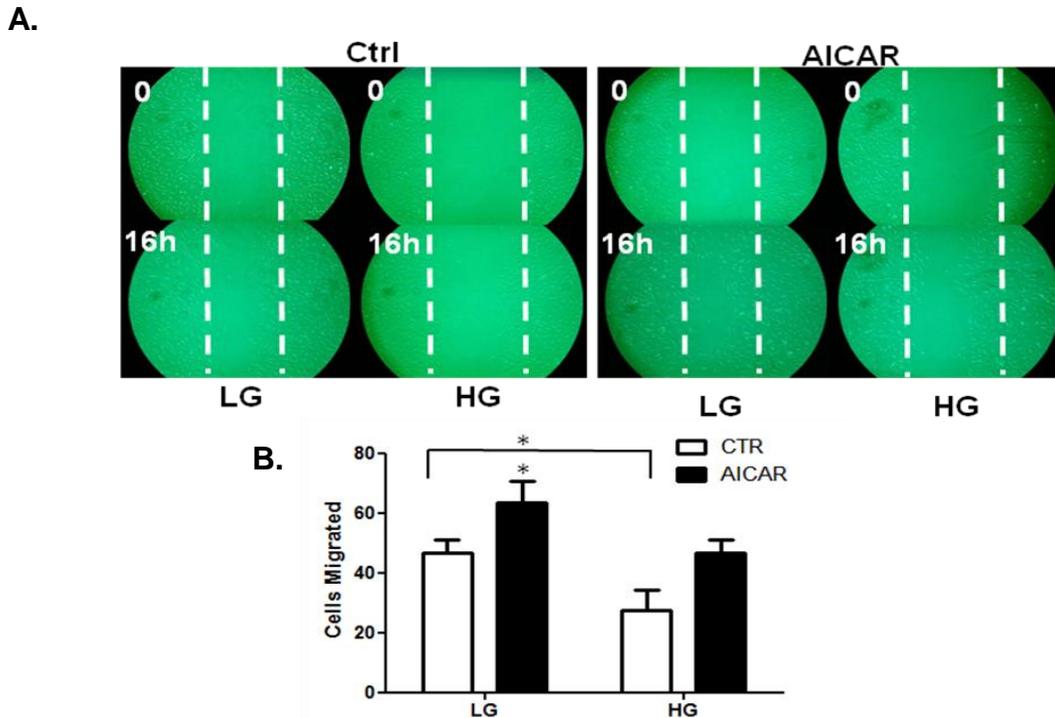


Figure 1. HG attenuation of EC migration is AMPK dependent.

(A) Representative photographs of EC monolayers scratched with a 1 ml pipette tip then incubated with LG (5.6 mM) or HG (25 mM) and treated with AICAR (1 mM) or control vehicle. (B) Bar graph quantification of scratch assay results shown as mean \pm SEM of eight independent experiments. * indicates $P < 0.05$.

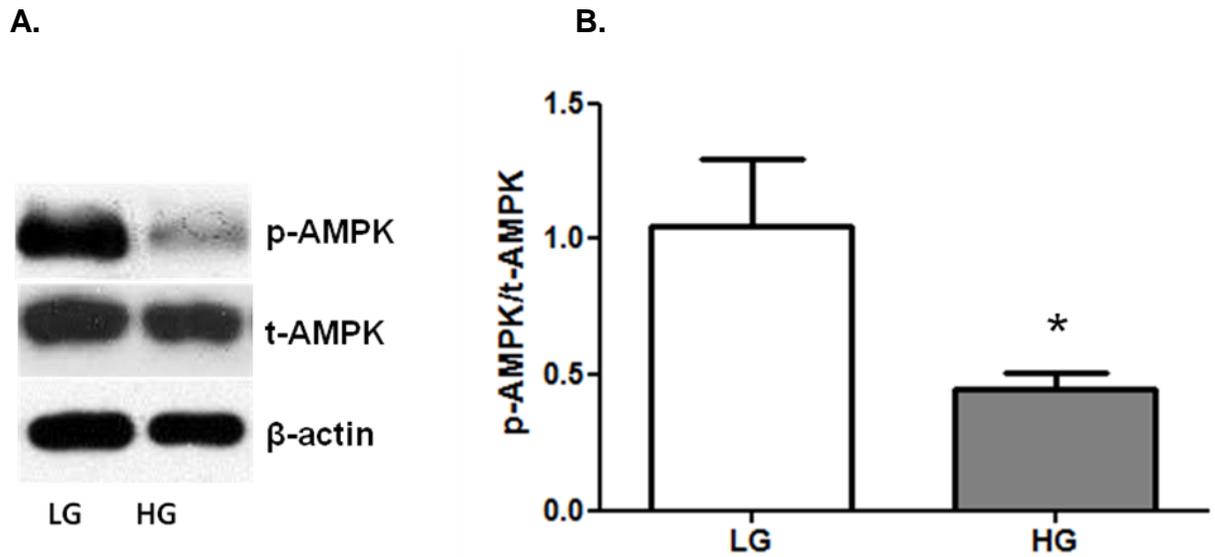
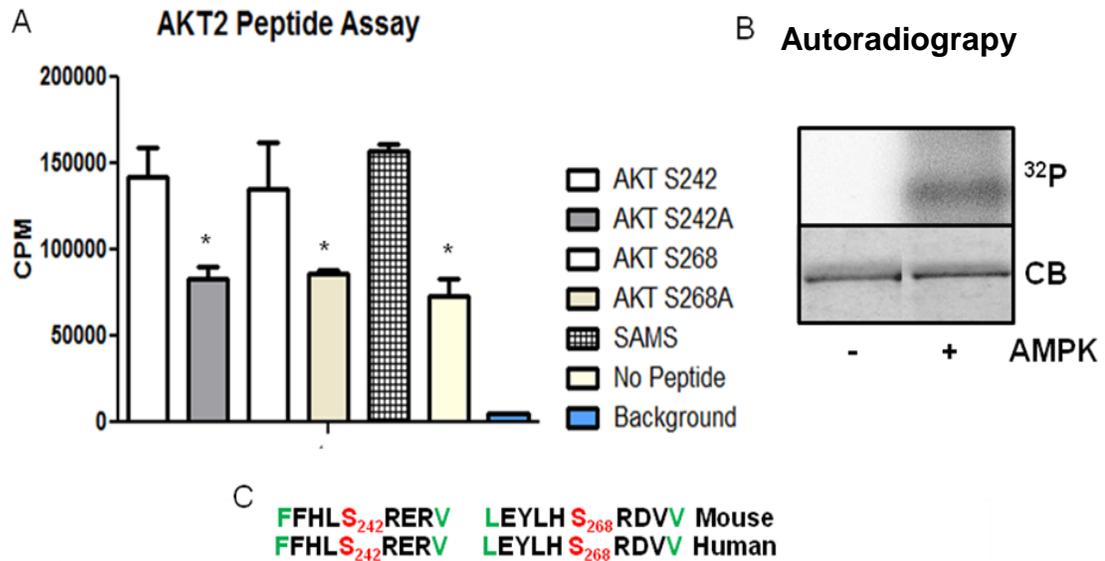


Figure 2. HG-attenuates level of phosphorylated p-AMPK (T172).

(A) Representative immunoblot of endothelial cells cultured under LG (5.6mM) or HG (25mM) conditions for 24 hours. (B) Bar plot summarizing densitometry results of p-AMPK to t-AMPK shown as the mean \pm SEM of eight independent experiments, ratio under LG conditions set at a value of 1. * $P < 0.05$.



AMPK phosphorylates consensus motif (L/M)XRXX(s/t)XXXL

D. Full length flanking peptides

AKT2 S242 ELFFHLSRERVFTRR
 AKT2 S242A ELFFHLARERVFTRR
 AKT2 S268 ALEYLHSRDVVYRRR
 AKT2 S268A ALEYLHARDVVYRRR

Fig. 3. AMPK phosphorylates AKT2.

(A) Bar graph summarizing in-vitro peptide kinase activity assay using AKT2 peptides (15-mers) that include S242 or S268. (B) Autoradiograph of a mixture of [³²P] γ-ATP and recombinant full-length AKT2 protein with or without recombinant AMPK. Coomassie blue (CB) stained. (C) the peptide sequence surrounding the putative phosphorylation sites in aligned mouse and human AKT2 sequence. (D) The peptide sequence used for AKT2 assay. Results are mean ± SEM of three independent experiments. An “*” indicates p < 0.05.

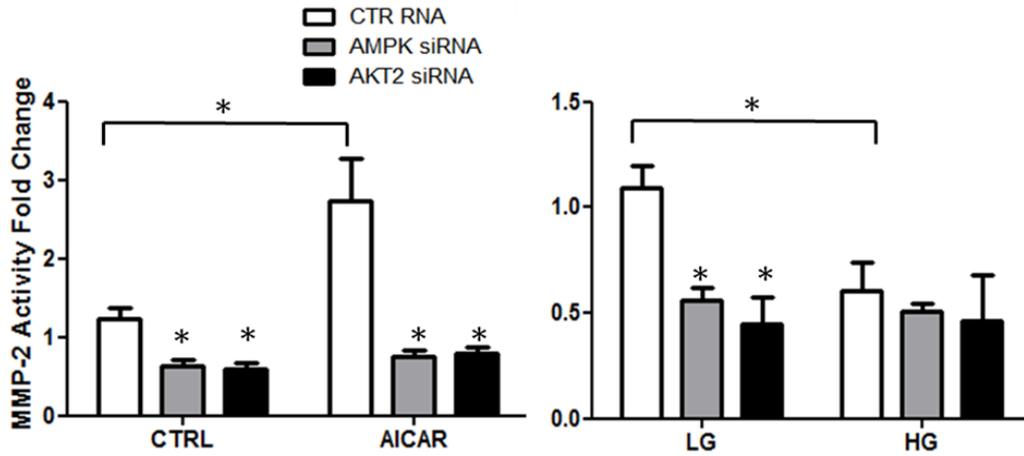
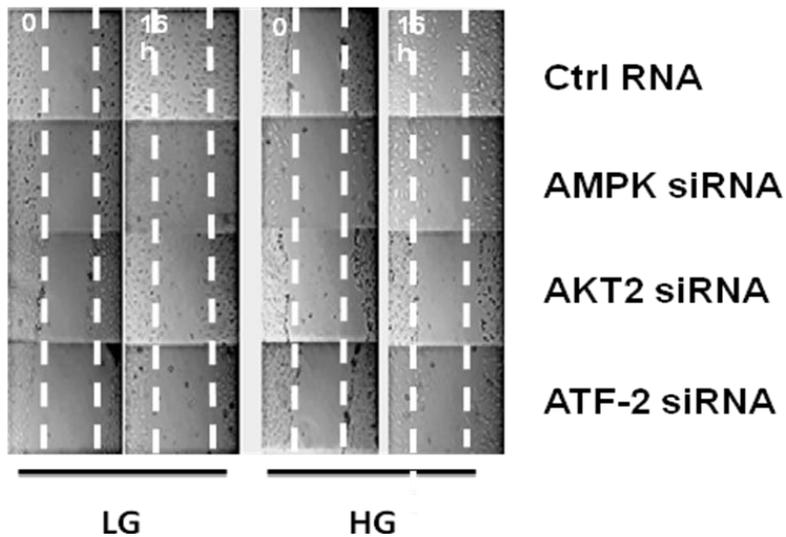


Figure 4. MMP-2 activity is dependent on AMPK and AKT2.

(A) Bar plot summarizing MMP-2 activity. ECs grown in HG were transfected with AMPK siRNA , AKT2 siRNA or control (Ctrl) siRNA, then treated with AMPK activator (AICAR, 1 mM) or vehicle control. (B) Bar graph summarizing results of ECs transfected as above and cultured in LG or HG. ‘*’ indicates $p < 0.05$.

A.



B.

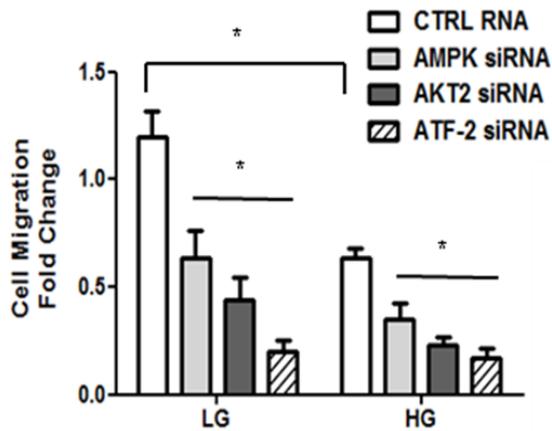


Figure 5. HG reduced EC migration is dependent on the AMPK-AKT2-ATF-2-MMP-2 pathway. (A) Photomicrographs of scratched EC monolayers transfected with siRNA (AMPK, AKT2, ATF-2 or Ctrl). (B) Summary the results of 3 independent experiments. Cell migration fold change as a function of siRNA transfections and D-glucose. LG /Ctrl conditions. ‘*’ indicates $p < 0.05$.

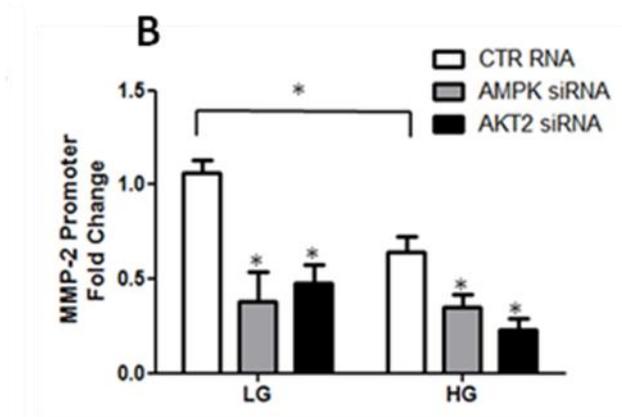
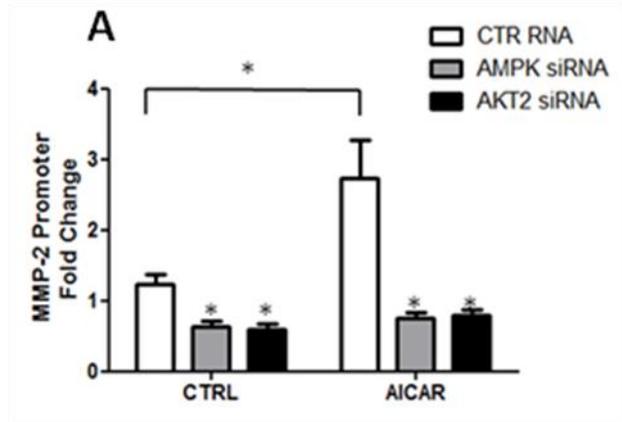


Figure 6. AMPK and AKT2 are necessary for ATF-2 binding to the MMP-2 promoter. (A) Bar plot summarizing the effect of AMPK activation on ATF-2 binding to MMP-2 promoter, (B) Bar graph summarizing the negative effect of HG on ATF-2 to MMP-2 binding (Fig. 6B). ‘*’ indicates $p < 0.05$.

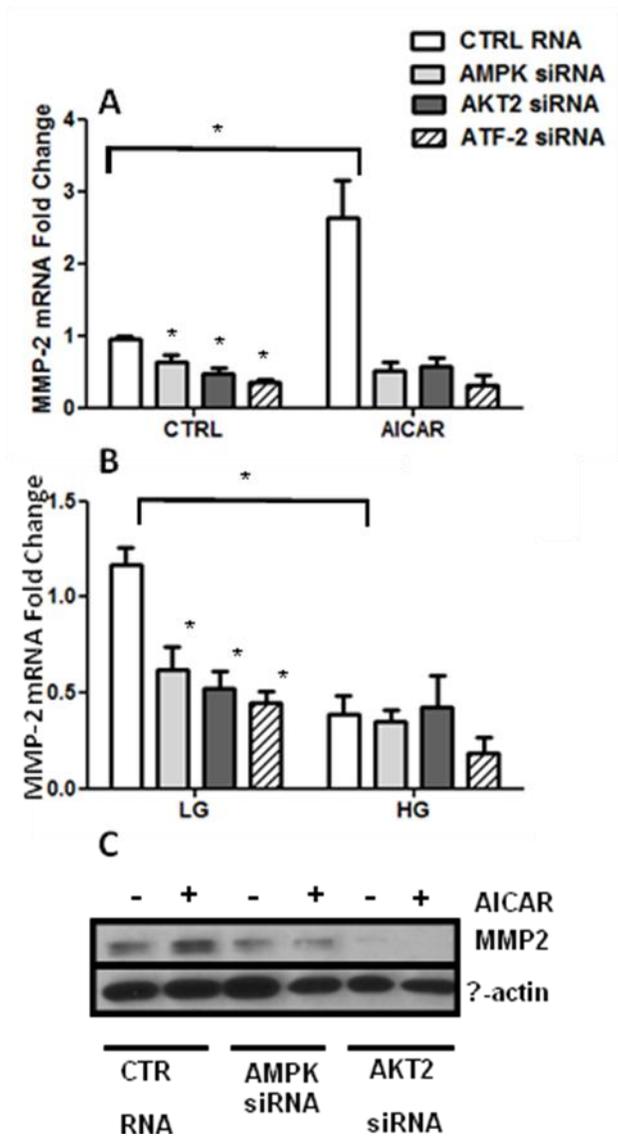


Figure 7. AMPK, AKT2 and ATF-2 are necessary for AICAR and LG induced MMP-2 expression. (A) MMP-2 messenger RNA (mRNA) in ECs transfected with siRNA (AMPK, AKT2, ATF-2 or Ctrl) and treated with AICAR or vehicle control or (B) grown in LG or HG. (C) Immunoblot of the effects of AMPK activation and AKT-2 and AMPK knockdown on MMP-2 protein levels. Results expressed as mean \pm SEM. “*” indicates $p < 0.05$.

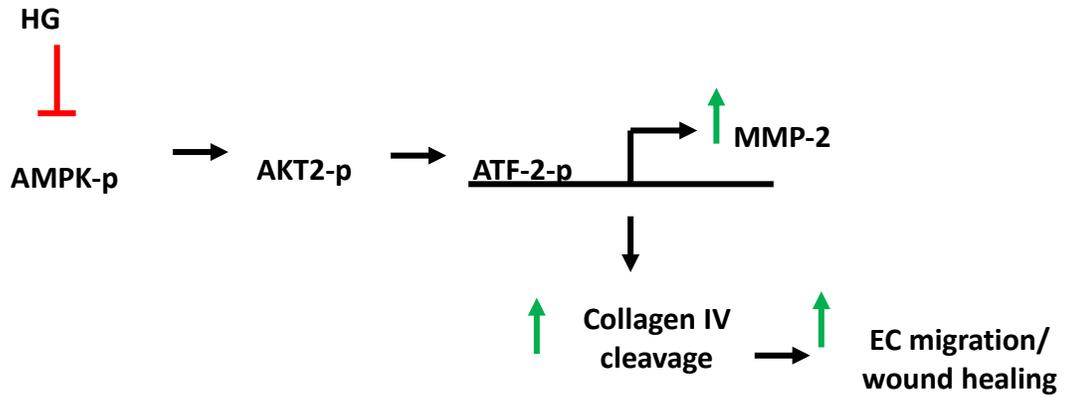


Figure 8. Schematic model of HG-impaired EC migration. High glucose inhibits AMPK activation, subsequently decreasing AKT2 phosphorylation and activation and its downstream substrate ATF-2. ATF-2 is a transcription factor for MMP-2. MMP-2 cleaves collagen IV to facilitate EC migration.

3.0 Perspectives and Conclusions

Hyperglycemia, or high blood sugar, is a condition in which an excessive amount of glucose circulates in the blood plasma. Primary prevention of the vascular complications of HG, ie, impaired EC migration and wound healing is to avoid or minimize those conditions causing acute or chronic HG whenever possible. Increased longevity and improved health can be achieved in mammals by two feeding regimens, caloric restriction (CR), which limits the amount of daily calorie intake, and intermittent fasting (IF), which allows the food to be available ad libitum every other day. Intermittent fasting can alternatively be less rigorous, eg, 1 or 2 meals per day instead of 3. Caloric restriction (CR), is a dietary regimen that restricts calorie intake, where the baseline for the restriction varies, usually being the previous, unrestricted, intake of the subjects. Calorie restriction without malnutrition has been shown to improve age-related health and to slow the aging process (including vascular aging) in a wide range of animals.⁷⁹⁻⁸⁰

Energy restriction (ER) to control weight is a potential strategy to prevent some forms of cancer, insulin resistance and hyperglycemia. Frequent spikes in HG caused by the consumption of excess simple carbohydrates, with a high glycemic index, eg, sugar sweetened beverages.⁸²

Nutriceuticals, eg resveratrol (RSV), β -carotene, vitamins C that are known to have anti-oxidant properties can help neutralize the HG induced

oxidative stress in ECs. In addition RSV activates AMPK in ECs. Chronic resveratrol administration has beneficial effects in experimental model of type 2 diabetic rats. Four-month oral resveratrol administration (5 mg/kg/day) significantly attenuated the elevated levels of the blood glucose, glycosylated hemoglobin in diabetic rats. Moreover, resveratrol administration to diabetic rats improved the reduced levels of glutathione, total antioxidant capacity, and the antioxidant enzymes activities (superoxide dismutase, glutathione peroxidase, and catalase).⁸¹

Medications that activate AMPK can potentially rescue some of the EC migratory capacity that was diminished by HG conditions. Statins and metformin activate AMPK in ECs. Metformin inhibits hepatic gluconeogenesis, thus decreasing circulating glucose levels, and it increases insulin sensitivity. Intracellularly, metformin activates AMPK.⁸³⁻⁸⁴

Increased energy expenditure by large muscle, lower extremity aerobic exercise can increase skeletal muscle glucose uptake, lower plasma glucose, increase shear stress, activate AMPK and increase foot and ankle perfusion and wound healing in the dependent limbs.⁸⁶

4.0 References

1. American Association of Diabetes Clinical Guidelines. URL: <http://professional.diabetes.org/ResourcesForProfessionals.aspx?cid=84160>
2. Coutinho M, Gerstein HC, Wang Y, Yusuf S. The relationship between glucose and incident cardiovascular events. A metaregression analysis of published data from 20 studies of 95,783 individuals followed for 12.4 years. *Diabetes Care* 1999;22:233–40.
3. de Vegt F, Dekker JM, Ruhe HG, Stehouwer CDA, Nijpels GBLM, Heine RJ. Hyperglycaemia is associated with all-cause and cardiovascular mortality in the Hoorn population: the Hoorn Study. *Diabetologia* 1999;42:926–31.
4. Donahue RP, Abbott RD, Reed DM, Yano K. Postchallenge glucose concentration and coronary heart disease in men of Japanese ancestry. Honolulu Heart Program. *Diabetes* 1987;36:689–92.
5. Lowe LP, Liu K, Greenland P, Metzger BE, Dyer AR, Stamler J. Diabetes, asymptomatic hyperglycaemia, and 22-year mortality in black and white men. The Chicago Heart Association Detection Project in Industry Study. *Diabetes Care* 1997;20:163–9.
6. The DECODE study group on behalf of the European Diabetes Epidemiology Group. Glucose tolerance and mortality: comparison of WHO and American Diabetes Association diagnostic criteria. *Lancet* 1999;354:617–21.
7. Balkau B, Shipley M, Jarrett RJ, et al. High blood glucose concentration is a risk factor for mortality in middle-aged non diabetic men. 20-year follow-up in the Whitehall Study, the Paris Prospective Study, and the Helsinki Policemen Study. *Diabetes Care* 1998;21:360–7.
8. Hanefeld M, Fischer S, Julius U, et al. Risk factors for myocardial infarction and death in newly detected NIDDM: the Diabetes Intervention Study, 11-year follow-up. *Diabetologia* 1996;39:1577–83.
9. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature* 2001;414:813–20.
10. Brownlee M. The pathophysiology of diabetic complications: a unifying mechanism. *Diabetes* 2005;54:1615–25.
11. Ceriello A, Quagliano L, Catone B, et al. Role of hyperglycemia in nitrotyrosine postprandial generation. *Diabetes Care* 2002;25:1439–43.

12. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* 1996;271:C1424–37.
13. Shishehbor MH, Aviles RJ, Brennan ML, et al. Association of nitrotyrosine levels with cardiovascular disease and modulation by statin therapy. *JAMA* 2003;289:1675–80.
14. Förstermann U, Münzel T. Basic Science for Clinicians: Endothelial Nitric Oxide Synthase in Vascular Disease. From Marvel to Menace *Circulation* 2006; 113: 1708-1714.
15. Vikramadithyan RK, Hu Y, Noh HL, Liang CP, Hallam K, Tall AR, Ramasamy R, Goldberg IJ. Human aldose reductase expression accelerates diabetic atherosclerosis in transgenic mice. *J Clin Invest* 2005: 2434–2443.
16. Wautier JL, Schmidt AM. Protein glycation: a firm link to endothelial cell dysfunction. *Circ Res* 2004; 95: 233–238.
17. Stitt AW, Li YM, Gardiner TA, Bucala R, Archer DB, Vlassara H. Advanced glycation end products (AGEs) co-localize with AGE receptors in the retinal vasculature of diabetic and of AGE-infused rats. *Am J Pathol* 1997; 150: 523–531.
18. Horie K, Miyata T, Maeda K, Miyata S, Sugiyama S, Sakai H, van Ypersele de Strihou C, Monnier VM, Witztum JL, Kurokawa K. Immunohistochemical colocalization of glycoxidation products and lipid peroxidation products in diabetic renal glomerular lesions: implication for glycoxidative stress in the pathogenesis of diabetic nephropathy. *J Clin Invest* 1997; 100: 2995–3004.
19. Goldin A, Beckman JA, Schmidt AM, Creager MA. Advanced glycation end products: sparking the development of diabetic vascular injury. *Circulation* 2006; 114: 597–605.
20. Xia P, Inoguchi T, Kern TS, Engerman RL, Oates PJ, King GL. Characterization of the mechanism for the chronic activation of diacylglycerol-protein kinase C pathway in diabetes and hypergalactosemia. *Diabetes* 1994; 43: 1122–1129.
21. Ayo SH, Radnik R, Garoni JA, Troyer DA, Kreisberg JI. High glucose increases diacylglycerol mass and activates protein kinase C in mesangial cell cultures. *Am J Physiol* 1991; 261: F571–F577.

22. Koya D, Jirousek MR, Lin YW, Ishii H, Kuboki K, King GL. Characterization of protein kinase C beta isoform activation on the gene expression of transforming growth factor-beta, extracellular matrix components, and prostanoids in the glomeruli of diabetic rats. *J Clin Invest* 1997; 100: 115–126.
23. Pugliese G, Pricci F, Pugliese F, Mene P, Lenti L, Andreani D, Galli G, Casini A, Bianchi S, Rotella CM. Mechanisms of glucose-enhanced extracellular matrix accumulation in rat glomerular mesangial cells. *Diabetes* 1994; 43: 478–490.
24. Du XL, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, Wu J, Brownlee M. Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. *Proc Natl Acad Sci* 2000; 97: 12222–12226.
25. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 2000; 404: 787–790.
26. Stadelmann, WK; Digenis, AG; Tobin, GR. "Physiology and healing dynamics of chronic cutaneous wounds". *American journal of surgery* 1998;176 (2A Suppl): 26S–38S
27. Risau W. Mechanisms of angiogenesis. *Nature* 1997;386:671–4. 2.
28. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1995;1:27–31.
29. Greenhalgh, D.G. "The role of apoptosis in wound healing". *The International Journal of Biochemistry & Cell Biology* 1998. 30 (9): 1019–1030
30. Lansdown, A.B.G.; Sampson, B.; Rowe, A. "Experimental observations in the rat on the influence of cadmium on skin wound repair". *International Journal of Experimental Pathology* 2001. 82 (1): 35–41
31. Ebrahimian TG, Heymes C, You D, Blanc-Brude O, Mees B, Waeckel L, Duriez M, Vilar J, Brandes RP, Levy BI, Shah AM, Silvestre JS. NADPH oxidase-derived overproduction of reactive oxygen species impairs postischemic neovascularization in mice with type 1 diabetes. *Am J Pathol* 2006 Aug;169(2):719-28.

32. Waltenberger J, Lange J, Kranz A. Vascular endothelial growth factor-A-induced chemotaxis of monocytes is attenuated in patients with diabetes mellitus: A potential predictor for the individual capacity to develop collaterals. *Circulation* 2000 Jul 11;102(2):185-90.
33. Abaci A, Oğuzhan A, Kahraman S, Eryol NK, Unal S, Arinç H, Ergin A. Effect of diabetes mellitus on formation of coronary collateral vessels. *Circulation* 1999 y 4;99(17):2239-42.
34. Tie L, Li XJ, Wang X, Channon KM, Chen AF. Endothelium-specific GTP cyclohydrolase I overexpression accelerates refractory wound healing by suppressing oxidative stress in diabetes. *Am J Physiol Endocrinol Metab*. 2009 Jun;296(6):E1423-9. doi: 10.1152/ajpendo.00150.2009. Epub 2009 Mar 31.
35. Witte MB, Barbul A. Role of nitric oxide in wound repair. *Am J Surg*. 2002 Apr;183(4):406-12.
36. Frank S, Stallmeyer B, Kämpfer H, Kolb N, Pfeilschifter J. Nitric oxide triggers enhanced induction of vascular endothelial growth factor expression in cultured keratinocytes (HaCaT) and during cutaneous wound repair. *FASEB J*. 1999 Nov;13(14):2002-14.
37. McVeigh GE, Brennan GM, Johnston GD, McDermott BJ, McGrath LT, Henry WR, Andrews JW, Hayes JR. Impaired endothelium-dependent and independent vasodilation in patients with type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia*. 1992 Aug;35(8):771-6.
38. Loomans CJ, de Koning EJ, Staal FJ, Rookmaaker MB, Verseyden C, de Boer HC, Verhaar MC, Braam B, Rabelink TJ, van Zonneveld AJ. Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes. *Diabetes*. 2004 Jan;53(1):195-9.
39. Hong Ding H, Triggle CR. Endothelial Cell Dysfunction and the Vascular Complications Associated with Type 2 Diabetes: Assessing the Health of the Endothelium. *Vasc Health Risk Manag*. 2005 March; 1(1): 55–71.
40. Helisch A, Schaper W. Arteriogenesis: the development and growth of collateral arteries. *Microcirculation*. 2003; 10(1):83-97.
41. Gallagher KA, Liu ZJ, Xiao M, Chen H, Goldstein LJ, Buerk DG, Nedeau A, Thom SR, Velazquez OC. Diabetic impairments in NO-mediated endothelial progenitor cell mobilization and homing are reversed by hyperoxia and SDF-1 alpha. *J Clin Invest* 2007; 117(5):1249-59.

42. Semenza GL. Vasculogenesis, angiogenesis, and arteriogenesis: mechanisms of blood vessel formation and remodeling. *J Cell Biochem* 2007;102(4):840-7.
43. Rivard A, Silver M, Chen D, Kearney M, Magner M, Annex B, Peters K, Isner JM. Rescue of diabetes-related impairment of angiogenesis by intramuscular gene therapy with adeno-VEGF. *Am J Pathol* 1999;154(2):355-63.
44. Babaei S, Teichert-Kuliszewska K, Monge JC, Mohamed F, Bendeck MP, Stewart DJ. Role of nitric oxide in the angiogenic response in vitro to basic fibroblast growth factor. *Circ Res* 1998 18;82(9):1007-15
45. Tamarat R, Silvestre JS, Huijberts M, Benessiano J, Ebrahimian TG, Duriez M, Wautier MP, Wautier JL, Lévy BI. Blockade of advanced glycation end-product formation restores ischemia-induced angiogenesis in diabetic mice. *Proc Natl Acad Sci U S A*. 2003;100(14):8555-60.
46. Tepper OM, Galiano RD, Capla JM, Kalka C, Gagne PJ, Jacobowitz GR, Levine JP, Gurtner GC. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation* 2002;106(22):2781-6.
47. Deshpande NN, Sorescu D, Seshiah P, Ushio-Fukai M, Akers M, Yin Q, Griendling KK. Mechanism of hydrogen peroxide-induced cell cycle arrest in vascular smooth muscle. *Antioxid Redox Signal* 2002;4(5):845-54.
48. Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury: Part II: animal and human studies. *Circulatio*. 2003;108(17):2034-40.
49. Tojo T, Ushio-Fukai M, Yamaoka-Tojo M, Ikeda S, Patrushev N, Alexander RW. Role of gp91phox (Nox2)-containing NAD(P)H oxidase in angiogenesis in response to hindlimb ischemia. *Circulation* 2005;111(18):2347-55. Epub 2005 May 2.
50. Simons M. Angiogenesis, arteriogenesis, and diabetes: paradigm reassessed? *J Am Coll Cardiol* 2005;46(5):835-7.
51. Tan AS, Ahmed N, Berridge MV. Acute regulation of glucose transport after activation of human peripheral blood neutrophils by phorbolmyristate acetate, fMLP, and granulocyte-macrophage colony-stimulating factor. *Blood* 1998;91(2):649-55.
52. Kukidome D, Nishikawa T, Sonoda K, Imoto K, Fujisawa K, Yano M, Motoshima H, Taguchi T, Matsumura T, and Araki E. Activation of AMP-activated protein kinase reduces hyperglycemia-induced mitochondrial reactive oxygen

species production and promotes mitochondrial biogenesis in human umbilical vein endothelial cells. *Diabetes* 55: 120-127, 2006.

53. Chen Z, Peng IC, Sun W, Su MI, Hsu PH, Fu Y, Zhu Y, DeFea K, Pan S, Tsai MD, Shyy JY. AMP-activated protein kinase functionally phosphorylates endothelial nitric oxide synthase Ser633. *Circ Res.* 2009;104(4):496-505.

54. Nagata D, Mogi M, and Walsh K. AMP-activated protein kinase (AMPK) signaling in endothelial cells is essential for angiogenesis in response to hypoxic stress. *J Biol Chem* 278: 31000-31006, 2003.

55. Hadad SM, Appleyard V, Thompson AM. Therapeutic metformin/AMPK activation promotes the angiogenesis phenotype in the ERalpha-negative MDA-MB-435 breast cancer model. *Breast Cancer Res Treat.* 2009 ;114(2):391. doi: 10.1007/s10549-008-0016-3.

56. Stahmann N, Woods A, Spengler K, Heslegrave A, Bauer R, Krause S, Viollet B, Carling D, Heller R. Activation of AMP-activated protein kinase by vascular endothelial growth factor mediates endothelial angiogenesis independently of nitric-oxide synthase. *J Biol Chem* 2010;285(14):10638-52.

57. Li X, Han Y, Pang W, Li C, Xie X, Shyy JY, and Zhu Y. AMP-activated protein kinase promotes the differentiation of endothelial progenitor cells. *Arterioscler Thromb Vasc Bio* 2008; 1789-1795, 2008.

58. Wang XR, Zhang MW, Chen DD, Zhang Y, Chen AF. AMP-activated protein kinase rescues the angiogenic functions of endothelial progenitor cells via manganese superoxide dismutase induction in type 1 diabetes. *Am J Physiol Endocrinol Metab.* 2011; 300(6):E1135-45. doi: 10.1152/ajpendo.00001.2011.

59. Creighton J, Jian M, Sayner S, Alexeyev M, Insel PA. Adenosine monophosphate-activated kinase alpha1 promotes endothelial barrier repair. *FASEB J.* 2011;25(10):3356-65.

60. Irrcher I, Ljubcic V, Hood DA. Interactions between ROS and AMP kinase activity in the regulation of PGC-1alpha transcription in skeletal muscle cells. *Am J Physiol Cell Physiol* 2009;296(1):C116-23.

61. Bellacosa A, Testa JR, Moore R, Larue L. A portrait of AKT kinases: human cancer and animal models depict a family with strong individualities. *Cancer Biol Ther.* 2004;3(3):268-75.

62. Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ. Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *J Biol Chem* 2001;276:38349-52.

63. Garofalo RS, Orena SJ, Rafidi K, Torchia AJ, Stock JL, Hildebrandt AL, Coskran T, Black SC, BreesDJ, Wicks JR, McNeish JD, Coleman KG. Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB beta. *J Clin Invest* 2003;112:197–208.
64. Shiojima I, Walsh K. Role of Akt signaling in vascular homeostasis and angiogenesis. *Circ Res* 2002;90:1243–50.
65. Chen J, Somanath PR, Razorenova O, Chen WS, Hay N, Bornstein P, Byzova TV. Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. *Nat Med* 2005;11:1188–96.
66. Morales-Ruiz M, Fulton D, Sowa G, Languino LR, Fujio Y, Walsh K, Sessa WC. Vascular endothelial growth factor-stimulated actin reorganization and migration of endothelial cells is regulated via the serine/threonine kinase Akt. *Circ Res* 2000;86:892–6.
67. Peng XD, Xu PZ, Chen ML, Hahn-Windgassen A, Skeen J, Jacobs J, Sundararajan D, Chen WS, Crawford SE, Coleman KG, Hay N. Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. *Genes Dev* 2003;17:1352–65.
68. Fan Y, Gong Y, Ghosh PK, Graham LM, Fox PL. Spatial coordination of actin polymerization and ILK-Akt2 activity during endothelial cell migration. *Dev Cell* 2009;16(5):661-74
69. Ben-Yosef Y, Miller A, Shapiro S, Lahat N. Hypoxia of endothelial cells leads to MMP-2-dependent survival and death. *Am J Physiol Cell Physiol* 2005 Nov;289(5):C1321-31.
70. Monaco S, Sparano V, Gioia M, Sbardella D, Di Pierro D, Marini S, Coletta M. Enzymatic processing of collagen IV by MMP-2 (gelatinase A) affects neutrophil migration and it is modulated by extracatalytic domains. *Protein Sci* 2006;15(12):2805-15.
71. Kräling, B. M., Wiederschain, D. G., Boehm, T., Rehn, M., Mulliken, J. B. and Moses, M. A. (1999). The role of matrix metalloproteinase activity in the maturation of human capillary endothelial cells in vitro. *J. Cell Sci.* 112, 1599-1609.
72. Somerville RPT, Oblander SA, and Apte SS. Matrix metalloproteinases: old dogs with new tricks. *Genome Biol* 2003; 4: 216.1–216.11.

73. Visse R and Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 2003; 92: 827–839.
74. Bergman MR, Cheng S, Honbo N, Piacentini L, Karliner JS, and Lovett DH. A functional activating protein 1 (AP-1) site regulates matrix metalloproteinase-2 (MMP-2) transcription by cardiac cells through interactions with JunB-Fra1 and JunB-FosB heterodimers. *Biochem J* 2003; 369: 485–496.
75. Krishnamachary B, Berg-Dixon S, Kelly B, Agani F, Feldser D, Ferreira G, Iyer N, LaRusch J, Pak B, Taghavi P, and Semenza GL. Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. *Cancer Res* 2003; 63: 1138–1143.
76. Song H, Ki SH, Kim SG, Moon A. Activating transcription factor 2 mediates matrix metalloproteinase-2 transcriptional activation induced by p38 in breast epithelial cells. *Cancer Res*. 2006; 66(21):10487-96.
77. Din FV, Valanciute A, Houde VP, Zibrova D, Green KA, Sakamoto K, Alessi DR, Dunlop MG. Aspirin inhibits mTOR signaling, activates AMP-activated protein kinase, and induces autophagy in colorectal cancer cells. *Gastroenterology* 2012 Jun;142(7):1504-15.e3
- .
78. Zhu F, Zhang Y, Bode AM, Dong Z.. Involvement of ERKs and mitogen- and stress-activated protein kinase in UVC-induced phosphorylation of ATF2 in JB6 cells. *Carcinogenesis* 2004; 25(10):1847-52. Gallegher et al. Diabetes, cancer, and metformin: connections of metabolism and cell proliferation. *Ann NY Acad Sci*, 2011 DEC 1243:54-68.
79. Harvey, M. Et al. Energy restriction and the prevention of breast cancer. *Proc Nutr* 2012 May;71(2):263-75.
80. Froy, O. et al. Effect of feeding regimens on circadian rhythms: implications for aging and longevity. *Aging (Albany NY)*. 2010 Dec 11;2(1):7-27.
81. Soufi FG, Chronic resveratrol administration has beneficial effects in experimental model of type 2 diabetic rats. *Endocr Regul*. 2012 Apr;46(2):83-90.

82. Fegherazzi, G. Consumption of artificially and sugar sweetened beverages and incident type 2 diabetes in the European Prospective Investigation into Cancer and Nutrition cohort. *Am J Clin Nutr.* 10.3945/ajcn.2013.
83. Sun, W. et al. Statins Activate AMP-Activated Protein Kinase In Vitro and In Vivo. *Circulation*
84. Zhang, Y. et al. Metformin interacts with AMPK through binding to γ -subunit. *Mol Cell Biochem.* 2012 Sep;368(1-2):69-76.
85. National Institute for Diabetes, Digestive and Kidney Disorders
86. Narkar, V. et al. AMPK and PPARdelta agonists are exercise mimetics. *Cell.* 2008 Aug 8;134(3):405-15
87. World Health Organization
88. National Health and Nutrition Examination Survey, Center for Disease Control and Prevention
89. 2011 National Diabetes Fact Sheet
90. Guo, S. and DiPietro, L.A. Factors Affecting Wound Healing. *J Dent Res.* 2010 March; 89(3): 219–229.