An Investigation of the GAPDH/Siah1 Pathway in Human Retinal Pericyte Apoptosis

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To my parents, Sandra and Orlando, for their unconditional love and support

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TABLE OF CONTENTS

Page

DEDICATION ii

ACKNOWLEDGEMENTS iii

LIST OF TABLES vii

LIST OF FIGURES viii

LIST OF ABBREVIATIONS x

Chapter

I. Diabetic retinopathy

A. Diabetic retinopathy; background and significance 1
B. Current therapies for diabetic retinopathy 3
C. Molecular mechanisms of diabetic retinopathy 6
D. Pericyte cell death and diabetic retinopathy 13

II. GAPDH/Siah1 pro-apoptotic pathway

A. GAPDH/Siah1 pathway in non-ocular systems 19
B. GAPDH/Siah1 pathway in ocular systems 23

III. Methods

A. Human primary cell culture and treatment 25
B. Human retinal pericyte transfection 27
C. Nuclear isolation and western blot analysis 27
D. Immunoprecipitation assays 28
E. Immunocytochemical analysis 29
F. Nitric oxide synthase assay 29
G. S-nitrosylation western blot assay 30
H. Qualitative real time-PCR 31
I. Cell viability assay 31
J. Apoptosis measurements 32
K. *In vivo* retinal imaging 32
L. Statistics 33

IV. High glucose-induced apoptosis via the GAPDH/Siah1 pathway

A. High glucose induces Siah1 upregulation in human retinal pericytes 34

B. High glucose causes an increase in the association between GAPDH and Siah1 37
C. High glucose causes GAPDH nuclear translocation 39
D. High glucose causes hRP apoptosis 41

V. Inhibition of the GAPDH/Siah1 pathway prevents high glucose-induced HRP apoptosis

A. Pharmacologic inhibition of the GAPDH/Siah1 pathway with R-Deprenyl 46
B. Inhibition of the GAPDH/Siah1 pathway with Siah1 siRNA 52
C. Inhibition of the GAPDH/Siah1 pathway with GAPDH/Siah1 blocking peptides 57

VI. Tumor necrosis factor-alpha induction of the GAPDH/Siah1 pro-apoptosis pathway

A. TNFα causes Siah1 total protein upregulation and R-Deprenyl inhibits this upregulation 65
B. TNFα-induced GAPDH/Siah1 association 66
C. TNFα-induced hRP apoptosis 67

VII. Imaging retinal pericyte-specific GAPDH staining

A. Animal Models of DR 69
B. GAPDH nuclear translocation *in vivo* 70

VIII. Conclusions and future directions 73

APPENDIX

A. Immunocytochemistry of NG2 staining (red). Nuclei stained with DAPI in blue. 85

B. siRNA oligomer sequences. 86

C. Diagram of Thermo scientific pierce s-nitrosylation western blot kit 87

D. Taqman gene expression IDs used in qRT-PCR 88

E. Represent image of a C57Bl/6 mouse retinal digest 89

REFERENCES 90

LIST OF TABLES

Table **Page**

1. TAT-FLAG peptide sequence identification.....26

LIST OF FIGURES

Figure **Page**

1) Proposed mechanisms linking hyperglycemia to the pathogenesis of diabetic retinopathy 12

2) Proposed mechanism of action of the pro-apoptotic pathway GAPDH/Siah1 22

3) High glucose cause an upregulation of Siah1 total protein 36

4) High glucose leads to an increase in the association between GAPDH and Siah1 .. 38

5) High glucose causes GAPDH nuclear translocation 40

6) Immunocytochemical analysis of GAPDH nuclear translocation 41

7) High glucose causes human retinal pericyte apoptosis 42

8) High glucoses causes an increase in nitric oxide synthase (NOS) 45

9) Western blot analysis of S-nitrosylated proteins 45

10) Deprenyl inhibits high glucose-induced Siah1 total upregulation 47

11) Deprenyl inhibits high glucose-induced GAPDH/Siah1 association 49

12) Deprenyl inhibits high glucose-induced GAPDH nuclear translocation 50

13) R-Deprenyl prevents high glucose-induced human retinal pericyte apoptosis 51

14) Siah1 knock-down (KD) efficiency 52

15) Inhibition of the GAPDH/Siah1 pathway with Siah1 siRNA prevents high glucose-induced GAPDH/Siah1 association 53

16) Inhibition of the GAPDH/Siah1 pathway with Siah1 siRNA in hRP nuclear fraction53 54

17) Inhibition of the GAPDH/Siah1 pathway with Siah1 siRNA prevents high glucose-induced GAPDH nuclear translocation 55

18) Siah1 siRNA inhibits high glucose-induced caspase-3 enzymatic activity 56

19) TAT-FLAG Peptide Identification 58

20) GAPDH/Siah1 blocking peptides block high glucose-induced GAPDH/Siah1 association 60

21) Immunocytochemical analysis of GAPDH nuclear translocation 61

22) GAPDH/Siah1 blocking peptides inhibit high glucose-induced caspase-3 enzymatic activity 62

23) TNFα induces Siah1 total protein in GAPDH/Siah1 specific manner 65

24) TNFα induces GAPDH/Siah1 association 65

25) Siah1 siRNA and GAPDH/Siah1 peptides block TNFα-induced GAPDH/Siah1 association 66

26) LPS and TNFα cause hRP apoptosis 67

27) Retinal staining of GAPDH in pericytes 72

28) GAPDH/Siah1 complex in human retinal pericytes (hRP), human retinal microvascular endothelial cell (hRMEC) and human dermal fibroblast (hDF) 76

29) Deprenyl inhibits high glucose-induced p53 upregulation 80

30) GAPDH/Siah1 pathway in human retinal pericyte apoptosis 81

LIST OF ABBREVIATIONS

AGEs	Advanced glycation end products
Ang-1	Angiopoieten1
BRB	Blood-retinal barrier
DAG	Diacyglycerol
DAPI	40,6-diamidino-2-phenylindole
DCCT	Diabetes Control and Complications Trial
DM	Diabetes mellitus
DME	Diabetic macular edema
DMEM	Dulbecco's Modified Eagle Medium
DR	Diabetic retinopathy
EBM	Endothelial basal medium
EC	Endothelial cells
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAT	Fructose-6-phosphate-amidotransferase
GSH	Glutathione
HbA1c	Glycated hemoglobin
HBSS	Hank's buffered salt solution
hDF	Human dermal fibroblasts
hRMEC	Human retinal microvascular endothelial cell
hRP	Human retinal pericytes
IL-1β	Interleukin-1 beta
LPS	Lipopolysaccharide
MAO-B	Monoamine oxidase enzyme
MMTS	Methanethiosulfonate
NG2	Neuron glial 2
NLS	Nuclear localization signal
NO	Nitric Oxide
NOD	Non-obese diabetic
NOS	Nitric oxide synthase activity
NPDR	Nonproliferative diabetic retinopathy
P53	Tumor protein
PARP	Poly-(ADP-ribose)-polymerase
PBS	Phosphate-buffered saline
PDGFβ	Platelet-derived-growth factor receptor beta
PDR	Proliferative diabetic retinopathy
PKC	Protein kinase C
ROS	Reactive oxygen species
Siah1	Seven In Absentia Homolog 1
SODs	Superoxide dismutase
STZ	Streptozotocin
TBST	Tris-buffered saline with Tween 20

TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor
TNFR	TNFα receptor (1, 2)
TUNEL	Transferase-mediated dUTP nick end labeling
UKPDS	United Kingdom Prospective Diabetes Study
VEGF	Vascular endothelial growth factor
WB	Western blot

CHAPTER I

Diabetic retinopathy

A. Diabetic retinopathy; background and significance

Diabetes mellitus is a chronic, systemic disease that results from the deleterious effects triggered by high levels of glucose in the blood. DM impacts over 30 million Americans every year and is estimated to affect over 600 million people worldwide by 2035 [1]. Not only is diabetes a burden to the health and lifestyle of patients with this disease, but it is also a financial burden. In 2012 it cost over 240 billion dollars to provide care and treatment for diabetic patients in the United States [2].

There are two main types of diabetes; type 1, an autoimmune disorder and type 2, a metabolic disorder. Normally after a meal is consumed insulin is released from the pancreas to remove excess glucose from the bloodstream. In patients with type 1 diabetes, the pancreas is not able to produce enough insulin causing glucose levels to rise uncontrollably. Type 2 diabetes results from an inability to properly metabolize insulin [3]. In this case, the demand for insulin to remove excess glucose is too great and the pancreas is not able to keep up with the rise in the demand. Some patients with type 2 diabetes also develop insulin resistance, making it even more challenging to regulate high sugar levels in the blood. Thus, both types of diabetes result from an inability to properly regulate blood glucose levels. Most of the people that are diagnosed with diabetes are diagnosed with type 2. The main cause of type 2 diabetes is attributed to obesity and lack of exercise [4]. It is important to note that several genetic components have linked to the onset of type 2 diabetes [5]. On the other hand type 1 diabetes is mostly diagnosed in children and the cause of this type of diabetes still

remains an active area of research. It is estimated that about 5-10% of people with diabetes suffer from type 1 diabetes [6].

Some of the major complications that arise from chronic hyperglycemia are hypertension, dyslipidemia, and the topic of this dissertation, blindness. Diabetic retinopathy (DR) is the number one cause of blindness in working age Americans, blinding an estimated 12% of diabetic patients in each year [7]. In fact it is believed that 45% of Americans with diabetes will develop some form of retinopathy as time from diagnosis lengthens. About 20-25% of people with diabetes have vision loss after 10 years of uncontrolled blood glucose levels [8]. DR is characterized as a microvascular complication of diabetes that affects the blood vessels in the retina. Pathology of DR presents itself in distinctive stages. The early stages, known as mild and moderate non-proliferative DR (NPDR), are characterized by a remodeling of the retinal microvasculature. These remodeling events include thickening of the capillary basement membrane, increased vascular permeability, death of retinal pericytes and the formation of acellular, atrophied capillaries [9, 10]. Both of these early stages can contribute to a complication of DR known as diabetic macular edema (DME). DME results in the build up of fluid in the macula and is the leading cause of blindness among people with DR. In humans, the macula is the region of the eye responsible for sharp, straight-ahead vision [11]. Any leakage of fluid and/or blood into this area of the eye causes swelling of the macula resulting in blurry vision, and if left untreated, can lead to vision loss [12].

As the disease progresses into the later stages it is characterized as proliferative diabetic retinopathy (PDR), ischemic retinal foci release pro-angiogenic signals that stimulate growth of new blood vessels [8, 13]. The growth of these blood vessels itself is

not pathological but these new blood vessels tend to be abnormal, fragile and are exceptionally permeable. Consequently, they leak blood and fluid into the surface of the retina and into the vitreous humor that fills the eye. Another complication that arises at these advanced stages of DR is detachment of the retina. During the later stages of disease the formation of scar tissue and large amount of retinal neovascularization that has grown into the vitreous cavity of the eye can contract, causing the retina to lift. This pulling and lifting can result in retinal detachment and cause severe visual loss and blindness [14].

B. Current therapies for diabetic retinopathy

Several clinical trials have shown that systemic regulation of blood glucose is beneficial in slowing the progression of DR. For example, the Diabetes Control and Complications Trial (DCCT) demonstrated a 76% decrease in the rate of DR progression in patients with type 1 diabetes after controlled plasma glucose levels [15]. Another clinical trial (the United Kingdom Prospective Diabetes Study; UKPDS) demonstrated that glucose control slowed DR progression by 25% in patients with type 2 diabetes [16]. Also, more recently a study showed that in patients with type 2 diabetes tight control of blood glucose levels correlated with reduced progression of DR over the course of only four years [17]. Both the DCCT and UKPDS clinical trials demonstrated that systemic control of glucose delays the progression of DR, but even under the best control of glucose patients still develop DR [18]. Most importantly, there is no evidence that tight blood glucose control eliminates the need for more advanced invasive therapies. Therefore, researchers have been particularly interested in finding therapies

that address the early stages of the disease.

Currently, the gold standard for treatment of PDR is pan-retinal photocoagulation. This therapy has been the main option for patients with DR since it was first developed in 1985 [19]. During pan retinal photocoagulation small, high intensity laser burns are placed around the periphery of the retina. This causes an ablation of retinal cells leading to an increase in intraocular oxygen tension as oxygen utilizing cells are killed and a reduction in the production of pro-angiogenic factors as the cells that produce those factors, like vascular endothelial growth factor (VEGF), are killed. The main objective of this therapy is to prevent the formation of new pathological blood vessels. Several trials demonstrated a 50% reduction in vision loss from patients suffering from PDR when laser therapy is used [8, 20].

Although pan-retinal photocoagulation therapy is one of the main treatment options for patients with PDR there are several significant side effects associated with this treatment. For instance, laser therapy has been shown to worsen macular edema, impair night vision and damage peripheral retinal function [21]. Due to the invasiveness of this treatment, several trials have been conducted to minimize the negative side affects associated with photocoagulation. Most recently The PETER PAN study reveled that lower laser power settings and shorter duration of each burn resulted in the same outcome as traditional laser therapy [22]. These optimizations increase patient compliance and lower the cost and duration of each clinic visits. Although laser technology and clinical practices have greatly improved since laser therapy was first used in the late 1980s, this treatment is still highly invasive and burdensome to patients.

One of the most heavily investigated factors in the field of DR research is the

molecule known as VEGF. VEGF has been closely tied to the progression and severity of DR. Not only are VEGF levels significantly elevated in patients with diabetic macular edema when compared to healthy controls but VEGF has also been shown to directly alter blood-retinal barrier (BRB) integrity and stability [23, 24]. Currently, there are several drugs designed to specifically inhibit VEGF activity. Pegaptanib is an anti-VEGF aptamer, ranibizumab (Lucentis) is a monoclonal antibody fragment and bevacizumab (Avastin) is a full-length antibody targeted against VEGF [8]. Besides employing antibodies to block VEGF, other therapies exist to impede this potent player in DR pathology. For instance, the Eylea also known as the VEGF-Trap and Aflibercept, is a soluble VEGF receptor analogue. Both Lucentis and Eylea are FDA approved for treatment of DME. Avastin was originally approved for use in treating cancer patients but is now widely accepted to treat both DME and PDR [7].

Currently, anti-VEGF therapies are being used for the treatment of PDR and DME [25]. Treating PDR and DME patients with anti-VEGF treatment has shown great promise due to its efficacy in halting neovascularization and is used in great frequency to treat patients with advanced stages of DR [26, 27]. Presently, there is a clinical trial comparing the use of anti-VEGF therapy on patients with immediate or delayed laser therapy [28]. Although the FDA has not yet approved the use of anti-VEGF therapy as a treatment for PDR, it is routinely used experimentally in the clinic and is used more frequently than laser therapy.

Like most treatments currently available there are several drawbacks to using anti-VEGF therapy. Presently, anti-VEGF therapies are administered monthly via intravitreal injections. This method of delivery is burdensome to the patient and also

carries the risk of developing endophthalmitis [29]. Likewise, due to the frequency of these injections they become a burden not only to the patient but the health care provider as well. Intriguingly, anti-VEGF treatment has been shown to be effective in only a subset of patients with DME and additional concerns arise over the blockade of VEGF's neuro- and vasotrophic functions [8, 30]. Hence, the development of new and improved therapies against earlier stages of DR remains an important goal.

Lastly, there is growing evidence that inflammation plays a crucial role in the development of DR. Animal models of DR have shown increased vascular permeability, leukostasis, macrophage and neutrophil infiltration and as well as increased levels of pro-inflammatory cytokines such as tumor necrosis factor (TNFα) and VEGF [31]. Pro-inflammatory cytokines have also been shown to impact early disease progression by causing breakdown of the blood-retinal barrier [32]. Throughout the years several clinical trials have shown the beneficial effects steroids have on progression of DR [31, 33, 34]. Unfortunately, side effects, like the formation of cataracts and elevated intraocular pressure, have prevented the approval for steroid use in the United States [35]. Conversely, intravitreal inserts of fluocinolone acetonide are being administered as an effective DME treatment in the United Kingdom. The effects of steroids to treat neovascularization and PDR still remain unknown. Although steroids may be advantageous in treatment of later stages of diabetic retinopathy, laser therapy accompanied with anti-VEGF treatment remains the standard of care for DR.

C. Molecular mechanisms of diabetic retinopathy

Although there is a significant body of literature characterizing the later stages of

DR, the initial events that link hyperglycemia to the onset of DR still remain unidentified. That is why most of the therapies available to treat DR are aimed at the later more advanced proliferative stage of the disease. Further understanding of the early stages of DR has the potential to give rise to new therapies that prevent the progression of DR to more severe and less reparable stages.

There are four leading biochemical pathways that are hypothesized to link hyperglycemia to DR onset and progression. The first of these pathways is the polyol pathway. This pathway becomes activated when intracellular levels of glucose are elevated. Although cells use glucose as a primary source of energy, unused glucose enters the polyol pathway and is reduced to sorbitol and further converted into fructose [36]. During normal blood glucose levels this pathway causes no problems since it is successfully able to breakdown excess glucose into sorbitol and fructose. Under hyperglycemic conditions there is an imbalance in the proportions between sorbitol and fructose, and the reaction favors the production of sorbitol [37]. Sorbitol is an alcohol and is strongly hydrophilic. These biochemical properties trap sorbitol inside the cell since it is not able to cross the cell membrane. Consequently, sorbitol accumulates inside of the cells affecting osmotic pressure levels and causing oxidative stress [37]. Since retinal ganglion cells, Müller cells and vascular cells (endothelial cells and pericytes) all express aldose reductase, the enzyme that catalyzes the conversion of glucose into sorbitol, these cells are particularly susceptible to activation of the polyol pathway during diabetes [38, 39]. It is important to note that these cells are also the most heavily affected during the pathogenesis of diabetic retinopathy.

Investigation of the role of the polyol pathway in diabetic retinopathy progression

dates back to the early 1980s. Rat models of diabetes revealed compelling evidence that polyol pathway activation was a sufficient mechanism to explain the retinal abnormalities seen in DR [38, 40]. Similar studies conducted in diabetic mice failed to confirm this, and clinical trials to test the efficacy of polyol pathway inhibition have been inconclusive [41, 42].

The second molecular pathway hypothesized to play a role in hyperglycemia-induced DR progression is linked to the rapid increase in advanced glycation end (AGEs) product formation [36]. A glycation reaction refers to the addition of a carbohydrate to a protein. This non-enzymatic reaction occurs under normal conditions, but during diabetes there is an increase in the rate of AGE formation and accumulation. AGEs are particularly harmful to cells due to their ability to form crosslinks between proteins [43-45]. These modifications change protein structure and function and can cause permanent pathologic effects. Studies using animal models of diabetes have shown that long-term treatment with AGEs directly contributes to vascular complications [46]. Studies have also shown that treatment with AGE formation inhibitors (aminoguanide, vitamin B6 derivative pyridoxamine) reduces AGE accumulation, prevents the formation of microaneurysms, and prevents pericyte loss [45, 47]. Increased levels of AGEs have also been seen in retinal vessels of diabetic patients. There is also strong evidence correlating AGEs formation and accumulation with severity of DR [45, 48].

Recent efforts to treat early stages of DR by manipulating AGE formation/accumulation have centered on breaking AGE-induced protein crosslinks. For instance, an animal study using Alagebrium, a potent AGE-crosslink breaker, was

successful in inhibiting AGE induced protein crosslinking, activation of VEGF and accumulation of extracellular matrix products [48]. Future clinical trials must be conducted in order to access the ability of this treatment strategy to halt DR progression [45].

Another pathway that has been implicated in the progression of DR is activation of protein kinase C (PKC). PKC is a kinase involved phosphorylating hydroxyl groups of serine and threonine amino acid resides in signal transduction events including responding to hormonal, neuronal and growth factor stimuli. Although there are ten enzymes within the PKC family the β1/2 isoforms are the ones that appear to be particularly activated in diabetic vascular tissue [49]. In fact, expression of these isoforms is significantly enhanced in patients with diabetes [36]. PKC enzymes are activated by diacyglycerol (DAG). During diabetes, activation of the glycolysis pathway causes increased synthesis of DAG [50]. The PKC pathway is involved in numerous cell behaviors, and its activation has been shown to increase vascular cell proliferation, permeability, extracellular matrix production and production of pro-inflammatory cytokines in diabetic retinal tissues [49]. Thus, activation of the PKC pathway has direct consequences on DR progression by affecting both early events in DR pathology such as inflammation and later stages such as neovascularization [36, 51, 52].

The beneficial effects of inhibiting PKC activation have been demonstrated in both animal models of diabetes and in patients with DR [49, 53]. Specifically an inhibitor of PKC-β1/2 isoforms, ruboxistaurin (RBX; Eli Lilly), has been shown to reduce leukostasis and progression of DR [54]. Treatment with RBX was successful in reducing vision loss in patients suffering from DR but it was not able to prevent DR onset [55].

Since the PKC pathway is so heavily involved in normal physiology, investigators have urged caution in inhibiting the PKC pathway as a DR treatment. Ongoing research on this pathway will yield more information regarding the costs and benefits of inhibiting the PKC pathway in humans.

Lastly, increased activity of the Hexosamine pathway has also been linked the progression of diabetic retinopathy. Hexosamines are amino sugars that are created by addition of an amine group to a hexose. Hexosamine content is significantly increased in both animal models of DR and in patients with DR [56]. Also, several studies using animal models have shown that increased flux of glucose through this pathway plays a role in insulin resistance and diabetic vascular complications [56-58]. Other groups have also shown that increased flux of glucose through the hexosamines pathway results in neuronal apoptosis and neurodegeneration [59]. The initial step in the hexosamine pathway relies on a rate limiting step in which the fructose-6-phosphate-amidotransferase (GFAT) converts fructose 6-phosphate into N-acetylglucosamine-6-phosphate [60]. GFAT activity is closely correlated with increased glycated hemoglobin (HbA1c) levels in diabetic patients [61]. HbA1c levels are used to measure the average plasma glucose concentrations over long periods of times and is a way to measure blood glucose levels in patients with diabetes [62].

Although there has been extensive work elucidating the harmful effects of increased glucose flux through the hexosamine pathway in diabetes, little has been done to inhibit this pathway in the eye. Benfotiamine is a soluble lipid thiamine that has been shown to inhibit hexosamine production and decrease AGE formation in animal models of diabetes [63]. Hence, this agent might be a suitable tool to prevent the

progression of DR in humans.

All of four biochemical pathways discussed in this section result in or arise due to an increase in oxidative stress. Therefore, oxidative stress has been referred to as the “unifying mechanism” linking all destructive biochemical pathways associated with progression of DR [64]. Briefly, it is hypothesized that mitochondrial-derived reactive oxygen species (ROS) cause DNA damage. In order to repair this damage cells activate poly-(ADP-ribose)-polymerase (PARP) which inhibits glyceraldehyde phosphate dehydrogenase (GAPDH) activity causing an accumulation of glycolytic metabolites. These metabolites are then responsible for activation of AGEs, PKCβ2, polyol and hexosamine pathways [36, 64]. Specifically, oxidative stress is defined as a disproportion between the levels of oxygen radicals and antioxidant defenses [65]. The deleterious effects of oxidative stress have been considered hallmarks of chronic diseases like diabetes and cell death. Hyperglycemia induced oxidative stress has been characterized as a central step in microvascular complications associated with diabetes [66]. ROS target a wide range of macromolecules causing significant damage to cells and tissues resulting in the development and progression of retinopathy [67-69]. Under normal conditions ROS are detoxified by interacting with various reducing agents such as glutathione (GSH), vitamin E or reacting with enzymes such as superoxide dismutase (SODs), catalase and peroxide [70]. In hyperglycemic conditions, like the ones present in the retina of diabetic patients, availability of these reducing agents is significantly impaired [71]. Several animal studies have determined that oxidative stress not only contributes to the progression of DR but could explain the persistence of DR even under tight blood sugar control [72]. Unfortunately clinical trials using agents that

inhibit ROS formation have not proven to prevent progression of DR. Failure of these trials to work suggests that increased in oxidative stress is not be the only factor progression diabetic retinopathy to the late advanced stages. Therefore, the need to further investigate the steps linking hyperglycemia to disease progression is highly warranted. **Figure 1** depicts the role of oxidative stress in the molecular events hypothesized to occur during early stages of diabetic retinopathy.

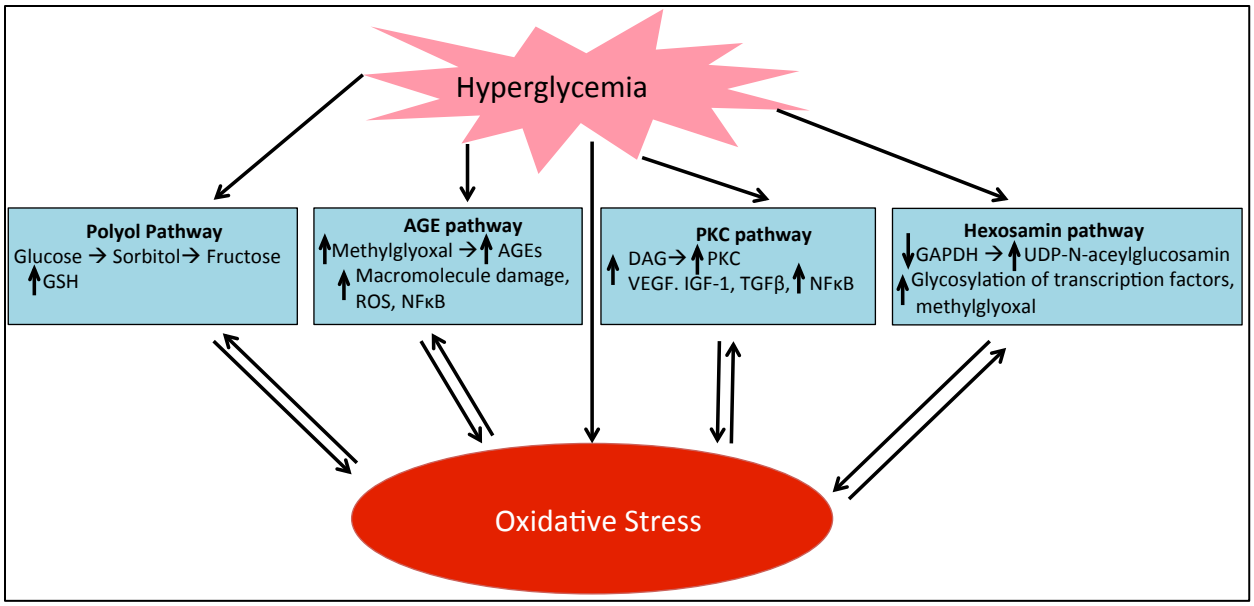


Figure 1. Proposed mechanisms linking hyperglycemia to the pathogenesis of diabetic retinopathy. This diagram depicts all major pathways hypothesized to play a role in the early events that connect high blood sugar levels with the onset and progression of disease. All of these pathways have been shown to be involved in oxidative stress production and have been shown to be activated by oxidative stress. Adapted from Kowluru et al. 2007 ref [67].

D. Pericyte cell death and diabetic retinopathy

Diabetic retinopathy is first characterized by a remodeling that occurs to the retinal vasculature. After thickening of basement membrane, by a mechanism that is still not fully understood, there is a selective loss of endothelial tight junctions. This results in the selective loss of intramural pericytes from retinal capillaries and is marked as one of the earliest histopathological changes that happens in DR [73]. Pericytes were first discovered in 1871 by Charles-Marie Benjamin Rouget and later further characterized by Zimmermann in 1923 [74, 75]. When they were first discovered, pericytes were described as perivascular cells that wrap around capillaries. Pericytes, often referred to as mural cells because of their contractile fibers, posses a cell body with a prominent nucleus and a small content of cytoplasm with long processes that embrace the endothelial surface [76]. These contractile cells are embedded within the basement membrane and are believed to regulate basement membrane assembly [77]. Pericyte processes allow the cells to contact neighboring ECs, integrating signals throughout the vessels. Pericyte to EC communication is essential for maintaining vascular stability and can occur through direct physical contact or through autocrine/paracrine signaling pathways. Direct physical contact occurs through peg-socket connections in which pericyte cytoplasmic fingers (pegs) are inserted into endothelial invaginations (pockets) [78]. They also can occur through the formation of adhesion plaques and gap-junction like structures [79, 80].

Although pericytes are primarily known to promote and stabilize hemodynamic process of blood vessels they play several roles *in vivo*. Some of these roles include sensing angiogenic stimuli, guiding sprouting tubes, promoting endothelial cell survival

and have also been shown to have macrophage like properties [81]. One of the ways that pericytes promote vascular stability is by forming intimate associations with endothelial cells (EC). Besides directly communicating with each other, pericytes and ECs are part of several vital signaling pathways. For example, platelet-derived-growth factor receptor beta (PDGF/PDGF-β) signaling is known to be important to the interaction between ECs and pericytes [76]. PDGF is released from EC and binds to its receptor, PDGF-β, expressed on the surface of developing pericytes. Activation of the PDGF/PDGF-β pathway plays a key role during embryonic development by controlling proliferation and differentiation of pericytes. For example, mice lacking pdgfb or pdgfβ have severe mural cell deficiency resulting in perinatal lethality [82, 83].

Another signaling mechanism that plays an essential role in pericyte/EC communication is the transforming growth factor beta (TGF-β) pathway. Both ECs and pericytes express TGF-β ligand and the TGFR-β receptor, but activation from the latent inactive form requires association between both cells. Since both cell types express both ligand and receptor, categorizing the role of each cell type in the TGF-β mechanism of action becomes fairly complicated. Numerous studies performed in cells and in animal models have demonstrated that TGF-β signaling plays a pivotal role in vascular development and pericyte differentiation and proliferation [78, 84]. In order to promote vascular development and stability pericytes and endothelial cells communicate using the TGFR-β2 receptor. TGFR-β2 signals are then transduced through a pair of transmembrane serine/threonine kinases known as activin receptor-like kinase (ALK) -1 and -5 [85]. Alk-5 has been shown to be responsible for promoting vessel quiescence and differentiation into smooth muscle cells. Conversely, Alk-1

promotes proliferation and migration and inhibits smooth muscle differentiation [86-89]. During early stages of TGF-β stimulation Alk-1 signaling is dominant, but after longer stimulation of TGF-β Alk-5 signaling dominates, leading to cell differentiation and extracellular matrix formation [76].

The angiopoieten1 (Ang-1)/tie-2 signaling pathway is another mechanism known to play a crucial role in pericyte-endothelial cell communication. In this system Ang-1 is expressed by pericytes, while the receptor, Tie-2, is expressed on ECs [90-92]. This pathway has been shown to mediate endothelial maturation and stability as well as control vascular leakage [93]. The importance of this pathway has been shown in genetically modified mice that lack Ang-1 or Tie-2. These mice are embryological lethal due to severe defective angiogenesis that results in inadequate blood vessel formation. These mice also have severely impaired formation of basement membranes and have significantly reduced number of pericytes [94-96].

Pericyte density greatly varies depending location of the pericytes. Retinal digest preparations have shown that in the retina pericytes and ECs exist in a 1:1 ratio. Pericyte coverage in the central nervous system is generally higher than most other organs and tissues [97, 98]. Pericyte density is believed to directly correlates with endothelial barrier properties. This strongly suggests that pericytes play key role in regulating capillary barrier stability [76].

Although the specific mechanism responsible for causing pericyte loss from the vasculature during diabetes remains unknown, it is known that these cells die via an apoptotic mechanism. Apoptotic cell death during early stages of diabetic retinopathy has been observed in several animal models of diabetes [99, 100]. For instance, one

group using the streptozotocin-induced (STZ) diabetic mouse model demonstrated a significant increase in retinal vascular apoptosis [101]. Since this study was performed, several other investigators using the STZ and db/db diabetic models have also shown a significant increase in retinal vascular cell apoptosis in diabetic animals when compared to healthy controls [102-104]. Not only do retinal vascular cells die in animal models of diabetes via an apoptotic mechanism, but apoptosis is also seen in human diabetic retinas. One study conducted by Mizutani et al. demonstrated an increase in transferase-mediated dUTP nick end labeling (TUNEL) in vascular cells from human diabetic retinas when compared to non-diabetic controls [100]. There is also evidence from postmortem diabetic retinas suggesting that the number of pericytes undergoing apoptosis is greater than that of endothelial cells [99]. Another study demonstrated a significant increase in Bax expression in diabetic patients as well as *in vitro* and *in vivo* models of diabetes [105]. Both TUNEL staining and Bax expression are markers commonly used to identify apoptotic cell death. Retinal pericyte apoptosis is also evident by the formation of “pericyte ghosts”. The formations of these “ghosts” are commonly seen in retinal samples from diabetic animals and patients. Pericyte ghosts are pockets within the basement membrane that outline where a pericyte once existed [101]. Since one of the primary roles of retinal pericytes is to promote vessel stability and health, once these cells are gone from the vasculature, the integrity of the vessel is severely compromised.

Due to the tight and highly controlled crosstalk between endothelial cells and pericytes it is clear to see how loss of pericytes could in turn result in the later loss of endothelial cells [73, 106]. It is important to note that although there is ample evidence

showing the loss of pericytes occurring before the loss of endothelial cells all of these human studies are done in postmortem processed tissue and due to the difficulty to acquire human samples; sample sizes tend to be small [104]. As the disease progresses and more cells are lost from the vasculature vessels become hypoxic and release pro-angiogenic signals that stimulate growth of new vessels. These new blood vessels provide the essential nutrients required for proper retinal functions [104]. As previously stated, these vessels tend to be abnormal and cause progression of the disease into the threatening proliferative stage.

Although it is widely recognized that pericytes undergo apoptosis early in DR, the molecular mechanism(s) that mediate this event during early stages of DR remain(s) unknown. Most of the mechanisms of DR progression explained in **Chapter 1 Section D** have been implicated to play a role in pericyte apoptosis as well. For example, several groups have found a significant increase in bovine pericyte apoptosis as a result of treatment with AGEs [107, 108]. AGE overexpression has also been shown to cause a dramatic increases in VEGF mRNA expression, suggesting that AGEs are not only able to induce pericyte apoptosis but are also able to induce VEGF overproduction leading to the pathogenesis seen in early and later stages of DR [107]. Exposing bovine pericytes to AGEs also resulted in an increase is osmotic stress. Since the retina has one of the highest oxygen uptake and glucose oxidation consumption rates, it becomes especially susceptible to increases in reactive oxygen species produced by increase in oxidative stress [109].

Other pathways that have been implicated in pericyte apoptosis are the PKC and Polyol pathways. Geralde, et al. has demonstrated that hyperglycemia causes pericyte

apoptosis by activating the PKC pathway. Their study showed that activation of this pathway caused dephosphorylation of the PDGFβ receptor, which in turn resulted in pericyte-specific apoptosis [110]. Similarly, other groups have demonstrated in both animal models of DR and in diabetic patients that high glucose correlated with increases in the Polyol pathway resulting in pericyte cell death [38, 111]. Although all of these pathways have been implicated to play a role in early pericyte apoptosis there are currently no treatments available to prevent progression of DR. Therefore, further understanding on the molecular mechanisms responsible for hyperglycemia induced human retinal pericyte apoptosis are necessary.

CHAPTER II

GAPDH/Siah1 pro-apoptotic pathway

A. GAPDH/Siah1 pathway in non-ocular systems

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is conventionally known as a housekeeping gene due to its constant expression levels throughout various tissues and conditions. The primary role of this enzyme is to catalyze the sixth step of the glycolysis pathway. Specifically, GAPDH catalyzes the conversion of glyceraldehyde-3-phosphate to D-glycerate 1,3 biphoshate. GAPDH is composed of a tetrameric structure comprised of four identical 37kDa subunits. This enzyme has two functional domains, a catalytic site and a NAD^+ binding site [112]. The binding site is also known as the Rossmann fold which is believed to play a role in determining GAPDH's diverse localizations and functions. Studies have shown that GAPDH's catalytic function relies solely on two amino acids, $Cysteine^{152}$ and $Histidine^{179}$ [113].

Besides playing a key role during glycolysis GAPDH has been shown to have several important non-glycolytic functions as well. Some of these functions include gene transcription, RNA transport, DNA replication, endocytosis and apoptosis [112, 114-117]. Moreover, various studies have shown that during apoptosis in some cell types GAPDH translocates from the cytoplasm to the nucleus causing activation of programmed cell death pathways. This mechanism has been shown be to pro-apoptotic in HEK293 cells, S49 cells, primary thymocytes, PC12 cells, primary cerebral cortical neuronal cultures and in retinal glial cells [115]. GAPDH's role in apoptosis was first determined in 1996 by Ishitani et al. This group effectively demonstrated that GAPDH overexpression resulted in neuronal cell death and treatment with GAPDH antisense

oligonucleotide prevented this cell death [118]. Following this discovery the Sewa group investigated the mechanism of action by which GAPDH causes cell death. Since GAPDH does not have a nuclear localization signal (NLS) of its own and the GAPDH homo-tetramer is too large to freely enter the nucleus (molecular mass of 150,000) it heavily depends on another protein for nuclear entry [119]. Yeast two hybrid analysis determined that Seven In Absentia Homolog 1 (Siah1) was the sole binding partner with GAPDH out of a million clones [120].

Siah is known as the mammalian homolog of *Drosophila seven in absentia* (Sina) and is involved in photoreceptor development, specifically modulating R7 photoreceptor differentiation [121, 122]. In this system Sina forms a complex with Phyllopod and both proteins interact with Tramtrack, a transcriptional repressor, causing polyubitquitination and proteasomal degradation [123]. Siah1 is one of the two highly conserved human homologs of Sina. Both homologs (Siah-1/2) are E3-ubiquitin-ligases involved in proteasome-mediated degradation of specific proteins. In the ubiquitin-proteosome pathway, E3 ligases accept ubiquitin from an E2 conjugating enzyme and then quickly transfer a ubiquitin molecule to the corresponding target [121]. Siah1 mediates E3 ligase activity by directly binding to substrates through its C-terminus substrate-binding domain or by utilizing its N-terminal RING-finger domain, which is responsible for proteolysis. Siah1 also contains two cysteine rich zinc finger domains [124]. Through its role as an E3 ligase, Siah1 activity has been linked to several biological processes such as development, neuronal plasticity, inflammation, angiogenesis and apoptosis [120, 121, 125-127]. Unlike GAPDH, Siah1 has a C-terminus NLS allowing it to localize in both the cytosolic and nuclear portions of the cell [128].

The same study that identified Siah1 as a GAPDH binding partner also identified the last 19 amino acids located at the C-terminus as critical and necessary for proper binding to GAPDH [120]. Interestingly, mutating the amino acid Lysine^{225} results in complete abolishment of the interaction between GAPDH and Siah1 [120]. After stimulating neuronal (cerebellar granule neurons) and non-neuronal (RAW264.7, HEK293, N2a) cell types Hara et al. determined that cell stressors increase intracellular levels of nitric oxide (NO) which in turn cause S-nitrosylation of GAPDH. Due to the location of this post-translational modification (Cystein^{150}) GAPDH's catalytic activity is abolished and affinity for Siah1 binding is elevated. Being an E3 ligase, Siah1 has a high turnover rate [120]. This high turnover rate is significantly reduced when binding with GAPDH occurs. After binding, the complex is able to translocate to the nucleus where activation of the programed cell death pathway is initiated (**Figure 2**). Since the discovery of this pro-apoptotic pathway several groups have been interested in investigating the nuclear events resulting from nuclear translocation of the GAPDH/Siah1 pathway. It is important to note that the cytosolic events that lead to GAPDH/Siah1 binding have been carefully mapped out in several cell systems but the events that occur in the nucleus remain under intense investigation.

One of the mechanisms hypothesized to play a role in GAPDH/Siah1 associated cell death is the p300/CBP pathway. P300/CBP are the most prominent nuclear proteins acetyltransferase and has been shown to directly interact with GAPDH [129]. Nuclear

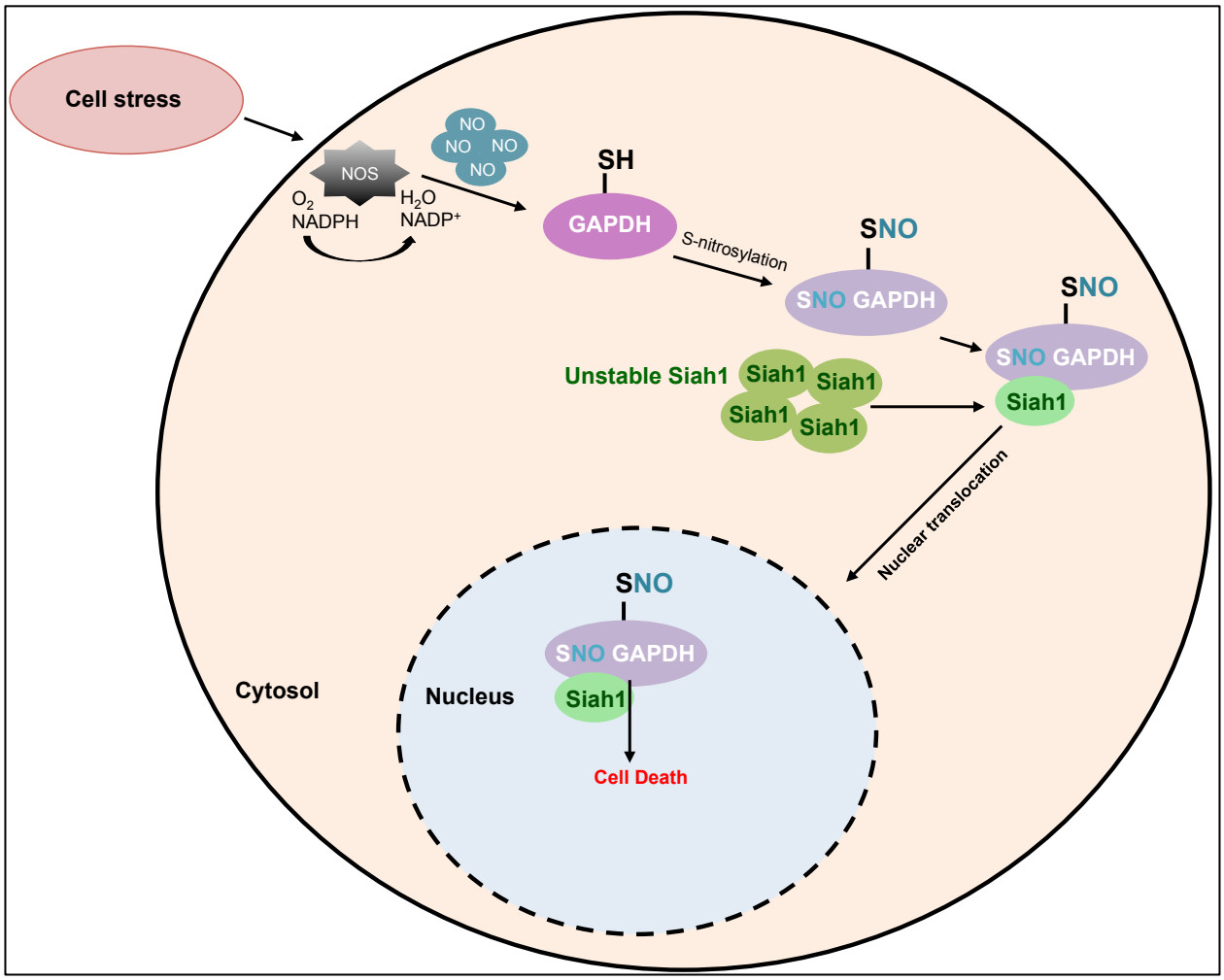


Figure 2. GAPDH/Siah1 pro-apoptotic proposed pathway. Proposed model of the pro-apoptotic pathway GAPDH/Siah1 in high glucose-induced human retinal pericyte apoptosis. Cell stress, such as high glucose, causes an increase in nitric oxide synthesis (NOS) activity. This increase in NOS activity results in elevated cytosolic nitric oxide (NO), which causes S-nitrosylation of GAPDH. Nitrosylated GAPDH associates with Siah1, stabilizing the complex and facilitating its translocation to the nucleus. Once in the nucleus, Siah1 degrades target proteins and/or GAPDH undertakes other non-glycolytic functions resulting in cell instability and ultimately cell death. This model is an adaptation from Hara et al. 2005. Ref [120].

acetylation creates a positive feedback loop resulting in elevated levels of p300/CBP activity. Acetylation of p300 leads to downstream activation of substrates like tumor protein (p53), resulting in cell death [129]. Also, Siah1 has been shown to upregulate p53, potentially linking directly the p53 cell death pathway with the GAPDH/Siah1 pro-apoptotic pathway [130]. More recent studies have shown that S-nitrosylated GAPDH leads to modification of other nuclear proteins such as sirtunin-1, histone deacetylase-2 and DNA activated protein kinase [131]. Modification of these nuclear targets could in turn lead to cellular instability and also result in cell death.

B. GAPDH/Siah1 pathway in ocular systems

It is widely understood that retinal cells undergo accelerated apoptosis during diabetic retinopathy but the specific molecular mechanisms that regulate this death still remains an active area of research [100]. Although no advances have been made in elucidating a role for the GAPDH/Siah1 pathway in retinal pericytes, there is evidence of the GAPDH/Siah1 pro-apoptotic pathway playing a role in retinal glial cell death [132].

Like pericytes, retinal glial cells are also affected by chronic exposure to high glucose in both *in vitro* and *in vivo* conditions [133]. Loss of retinal glial cells not only comprises tissue integrity but also leads to retinal degeneration [132]. Mohr, et al demonstrated that culturing retinal glial cells in high glucose caused cell death. This cell death is hypothesized to result from nuclear accumulation of GAPDH [134]. Later studies from this group also demonstrated that high glucose-induced GAPDH nuclear translocation is highly dependent on association and binding with Siah1 [124]. Moreover, treatment with high glucose also caused p53 phosphorylation, which could

explain the nuclear events occurring after the GAPDH/Siah1 complex translocates into the nucleus. Interestingly, inhibition of Siah1 with Siah1 siRNA inhibited high glucose-induced phosphorylation and subsequent cell death [124]. Thus, the work provided by this group suggests that nuclear accumulation of the GAPDH/Siah1 pro-apoptotic pathway accounts for the early events occurring during diabetes that lead to retinal glial cell death. Although there is some evidence that retinal glial cell death is important for DR progression, an extensive amount of evidence exists showing death of retinal vascular cells (pericytes and endothelial cells) at a much earlier event and loss of vascular cells significantly contributes to the progression of DR from the non proliferative to proliferative stages [135]. Thus, preventing earlier events of cell death like the ones seen in pericytes and endothelial cell death could be beneficial in also stopping the later death of glial cells.

After realizing that the GAPDH/Siah1 pathway plays a role in the death of one retinal cell type, it became apparent that this pathway needed to be studied in retinal pericytes. Since retinal pericyte death is one of the earliest events that occur in the pathogenesis of diabetic retinopathy, identifying ways to inhibit this cell death may lead to new strategies to retard or arrest progression of diabetic retinopathy. Although it has been previously shown that cell stress leads to nuclear accumulation of GAPDH resulting in an induction of cell death-related genes, the role of the GAPDH/Siah1 pathway in pericyte death has not been previously studied and is the subject matter of this dissertation.

CHAPTER III

Methods

A. Human primary cell culture and treatment

Primary cultures of human retinal pericytes (hRP) (Cell Systems; Kirkland, WA) were seeded into tissue culture flasks coated with attachment factor (Cell Signaling; Danvers, MA). HRP were grown and cultured in Dulbecco's modified Eagle's medium normal glucose (5.5mM DMEM 1X, Life Technologies; Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), and cell growth supplements, including antibiotics (Lonza; Basel). All cultures were incubated at 37°C, 5% CO_2 and 95% relative humidity (20.9% oxygen). Passages 5 to 7 were used for all experiments. HRP identity was confirmed by immunoreactivity of neuron glial 2 (NG2) (EMD Millipore; Temecula, CA) [136]. As further validation for NG2 as a pericyte specific marker, human retinal microvascular endothelial cells (hRMEC) and human dermal fibroblasts (hDF) were stained with anti-NG2 antibody. Out of the three cell types stained with NG2, hRPs are the only ones positive for NG2 (**Appendix A**). At 80% confluence hRP were treated with 10% FBS medium containing tumor necrosis factor alpha (TNFα; 10ng/ml), normal D-glucose (5.5mM), high D-glucose (25mM Sigma; St. Louis, MO) L-glucose (25mM Acros Organics; Geel, Belgium), which served as an osmotic control or R-deprenyl (1nM Tocris; United Kingdom). For TAT-FLAG peptide treatment, 1μM of control peptide, 1μM GAPDH peptide and/or 1μM Siah1 peptide was added to Hanks Balanced Salt Solution (HBSS; Life Technologies; Carlsbad, CA). Peptide solution was incubated at 37°C for 30mins before being added to each well. Cells were incubated with each peptide solution for 2hrs before experimental treatments were added. In cases where

peptides were used in combination, each original concentration was used for each peptide. Specific sequence identification of each TAT peptide can be found in **Table 1**. The N-terminal of each TAT-peptide is acetylated and the C-terminal is amidated; these modifications ensure proper cell entry and prevent degradation once inside the cell. A FLAG tag peptide sequence enables detection and quantification of these peptides.

Table 1. Peptide Sequence and Function		
Name	**Sequence**	**Function**
Control Peptide	N terminal acetylation- YGRKKRRQRRRDYKDDDDK-C terminal amidation	Positive cell entry control
GAPDH Peptide	N terminal acetylation-YGRKKRRQRRRDYKDDDDKVIPELNGKTLTGMAFRVPTA-C terminal amidation	Blocks GAPDH binding site on Siah1
Siah1 Peptide	N terminal acetylation-YGRKKRRQRRRDYKDDDDKGNLGINVTISMC-C terminal amidation	Blocks Siah1 binding site on GAPDH

Table 1. Peptide Sequence and Function. Each peptide was designed to competitively block the GAPDH binding site on Siah1 (GAPDH Peptide) or block the Siah1 binding site on GAPDH (Siah1 Peptide). The control peptide lacks specificity for either binding site; therefore this peptide serves as a positive control for proper cell entry.

Primary human retinal microvascular endothelial cells (hRMEC; catalog #ACBRI 181) were purchased from Cell Systems (Kirkland, WA) and grown in endothelial basal medium (EBM; Lonza; Walkersville, MD) with 10% fetal bovine serum (FBS; Atlanta Biologicals; Flowery Branch, GA) and endothelial growth supplements (EGM SingleQuots; Lonza). Cultures were kept in a humidified cell culture incubator at 37°C in 5% CO_2. HRMEC were treated with normal D-glucose (5.5mM), high D-glucose (25mM Sigma; St. Louis, MO), or L-glucose (25mM Acros Organics; Geel, Belgium), which served as an osmotic control.

Primary human dermal fibroblast (hDF; catalog number C-013-5C) were purchased from cascade biologics and grown in Medium 106 (Cat. No M-106-500) with 10% FBS plus antibiotics in the same incubation settings as hRP and hRMEC.

B*. Human retinal pericyte transfection*

For siRNA transfection, hRP were cultured in 6-well dishes and 1ml of fresh media was added to each well 30mins prior to treatment. For each well, 10μM siRNA oligomers (negative control siRNA or Siah1-directed siRNA) (**Appendix B**; siRNA sequence identification sc-37495A, B and C, Santa Cruz; Dallas, TX), 9μl Targefect Solution A (Targetingsystems; El Cajon, CA), and 18μl Virofect (Targetingsystems) were added to 250μl Optimem (Life Technologies) in a separate tube, and inverted between the addition of each reagent. Mixed reagents were incubated at 37°C for 25mins before being added to cultured hRP. Cells were incubated with transfection reagents for 12hrs, before being washed and treated with fresh media. Experimental treatments began 24hrs post-transfection.

C. Nuclear isolation and western blot analysis

HRP were grown in 10 cm dishes and treated as stated above. Cells were detached using TrypLE™ Express Enzyme (Life Technologies) and pelleted at 4000 x g in 4°C for 5 min. Lysates were washed with PBS and pelleted at 4000 x g in 4°C for 3 min. Nuclear and cytosolic fractions were collected using the NE-PER™ kit (Life Technologies) according to the manufacturer's directions in solutions containing protease (Halt™ Protease Inhibitor Cocktail; Thermo Scientific; Rockford, IL) and

phosphatase (Phosphatase Inhibitor Cocktail 2; Sigma-Aldrich) inhibitors. Protein concentration was determined using a bicinchoninic acid assay (BCA; Pierce; Rockford, IL). Protein samples were mixed with 4x Laemmli buffer (Bio-Rad; Hercules, CA) containing 355 mM β-mercaptoethanol (Bio-Rad) and then heated for 10 min at 96°C. 25 µg of protein was loaded onto a 10% SDS gel (Bio-Rad). The gel was run at 100 mV for 1 hr. The proteins were transferred onto a PVDF membrane (Life Technologies) using the iBLOT2 system (Life Technologies) P0 protocol (20 mV for 1 min, 23 mV for 4 min, 25 mV for 2 min). Membranes were blocked in 5% milk (for β-actin (Thermo Scientific) and GAPDH (Abcam; Cambridge, UK) immunoblots) or 5% BSA (for Siah1 (Santa Cruz), H3 (Cell Signaling), MEK (Cell Signaling) immunoblots) probed with appropriate primary antibody (anti-β-actin 1:3000, anti-GAPDH 1:1000, anti-Siah1 1:250, anti-Histone H3 and anti-MEK 1:750). Blots were then labeled with horseradish-peroxidase conjugated secondary antibodies diluted at 1:2000 (GAPDH, MEK and Histone H3; anti-rabbit, Siah1; anti-goat and β-actin; anti-mouse). MEK and Histone H3 served as cytoplasmic and nuclear fractionation control. β-actin was used to determine total protein concentration. Membranes were incubated in Pierce ECL western blotting substrate and developed using ChemiDoc MP (Bio-Rad; Hercules, CA). At least three independent experiments were used to generate western blot quantification graphs. Blots were quantified using the ImageJ 1.47v software.

D. Immunoprecipitation assays

HRP were treated as necessary and lysed using the Pierce IP lysis buffer. Equal amounts of protein (1000µg) from each sample were mixed with 10µg of anti-Siah1

antibody overnight at 4°C. Pierce Protein A/G Magnetic Beads were pre-cleared with Pierce IP lysis/wash buffer and added to the antigen sample/antibody mixture at room temperature for 1hr. Beads were collected with a magnetic stand and eluted using 50µl 4X SDS-PAGE reducing sample buffer at 100°C for 10mins. The immuno-complexes were then subjected to western blot analysis as described above. Siah1-depleted samples served as controls for total pull down of Siah1 from each lysate. Independent quality control experiments were performed in order to validate and confirm efficiency of the Siah1 immunoprecipitation.

E. Immunocytochemical analysis

HRP were cultured on multi-well glass slides and cells were permeabilized with 0.1%Triton-X100 in PBS for 30mins and blocked with 1.5% BSA in PBST overnight at 4°C. Cells were incubated with anti-GAPDH primary antibody (Abcam) overnight at 4°C. After incubation with primary antibody (1:100), cells were washed with PBST and incubated with secondary antibody for 1hr at room temperature. Cells were then washed in PBST and 40,6-diamidino-2-phenylindole (DAPI) stain was applied (Sigma) for 1min. Last, cells were washed and embedded using Fluorogel with Tris buffer (Electron Microscopy Science, Hatfield, PA, USA) and examined by fluorescence microscopy (Olympus AX70; Tokyo, Japan).

F. Nitric oxide synthase assay

Nitric oxide (NO) is a colorless gas that is highly unstable and has a short half-life. These properties make quantifying NO levels challenging. Nitrates and nitrites are

the products that result from the interaction between NO and molecular oxygen through activation of nitric oxide synthase. In order to measure levels of NO in hRP treated with disease relevant stimuli we used the nitric oxide synthase assay kit (EMD Millipore; Cat No. 482702). This kit relies on the natural biochemical events of NO production. Thus, this assay allows for the measurement of total levels of Nitrate (NO^3) and nitrites (NO^2). Manufactures instructions were followed as stated in kit manual. Samples were incubated with NAPDH and nitrate reductase solutions for 1hr at room temperature. Following this incubation, lactate dehydrogenase and additional cofactors were added as well for 20mins. Griess regents R1 and R2 were added to each sample and absorbance (540nm) was read using a plate reader 10mins later. Each sample absorbance is plotted at 540nm against each nitrate concentration. A nitrate standard curve ranging from 0μM-25μM is used to calculate each samples total nitrate concentration.

G. S-Nitrosylation western blot assay

The Thermo Scientific Pierce S-Nitrosylation western blot kit was used in order to measure levels of s-nitrosylation in hRP (work flow diagram shown in **Appendix C**). Cells were lysed with HENS buffer and free sulfhydryls are blocked with 1M Methanethiosulfonate (MMTS) for 30mins at room temperature. S-nitrocysteines were selectively reduced by adding 1uL of idoTMT labeling reagent and 20mg sodium ascorbate. Ultra pure water was used for negative controls instead of sodium ascorbate. Samples were reduced with 4X laemmli sample buffer and analyzed via western blot

analysis as described above. Anti-TMT antibody was diluted in 5%milk (1:1000) and anti-mouse IgG-HRP conjugated antibody was used as a secondary (1:20,000). measured 2hrs later.

H. Qualitative real time-PCR

RNA was reverse transcribed to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems; Carlsbad, CA) according to the manufacturer's directions. Quantitative real-time PCR (qRT-PCR) was performed by amplification of the gene of interest (*Siah1*) vs. *ACTB* (β-actin) using gene-specific TaqMan Gene Expression Assays (Applied Biosystems). Taqman gene expression IDs are found in **Appendix D**. Data were analyzed using the comparative Ct method and Ct values were normalized to *ACTB* levels.

I. Cell viability assay

Cell viability was measured using the CellTiter-Glo luminescent cell viability assay (Promega; Madison, WI). This assay determines cell viability based on the quantification of ATP levels present in each sample. Since ATP is a marker of metabolically active cells, the amount of ATP is proportional to the amount of viable cells in each sample. HRPs were treated with control, control peptide, GAPDH peptide or Siah1 peptide. As a positive control cells were cultured in 70% methanol. After background luminesce was determined, samples were equilibrated at room temperature for 30mins. CellTiter-Glo reagent (100µl) was added to each sample and luminesce was recorded after 15mins.

J. Apoptosis measurements

All apoptosis measurements were taken after 72hrs of appropriate treatment. Annexin V-FITC staining was one of the methods used to assay apoptosis. Cell pellets were resuspended in Annexin V binding buffer (Biolegend; San Diego, CA). Annexin V (Life Techonologies) and 7-AAD viability stain (Biolegend) was added to each sample for 15mins at room temperature. Samples were quantified using flow cytometry analysis performed at Vanderbilt's Flow Cytometry Shared Resource core laboratory. Apoptosis was also assayed by measuring Caspase-3 enzymatic activity. Activity was quantified using the EnzChek Caspase-3 Assay Kit (Life Technologies). Samples are incubated with 7-amino-4-methylcoumarin-derived substrate, Z-DEVD-AMC, for 1hr. Fluorescence emission was measured at 440nm.

***K. In vivo* retinal imaging**

All experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Six-week old male C57BL/6 mice were procured from Charles Rivers (Wilmington, MA). Mice received a 2µl intravitreal injection of vehicle (PBS), 50ng/ml TNFα or 10ng/ml LPS [137]. 24 hrs later, retinas were dissected and placed in HistoChoice fixative overnight. Retinas were permeabilized using 0.1%Triton-X100 in PBS for 30mins and blocked with 1.5% BSA in PBST overnight at 4°C. Retinas were then incubated with anti-GAPDH primary antibody (Abcam) and anti-NG2 antibody (Millipore) overnight at 4°C. After incubation with primary antibody (1:100), retinas were washed with PBST and incubated with

secondary antibody (1:100) for 1hr at room temperature. Retinas were then washed in PBST, embedded using Fluorogel with Tris buffer (Electron Microscopy Science, Hatfield, PA, USA) and examined by fluorescence microscopy (Olympus AX70; Tokyo, Japan).

L. Statistics

Data were analyzed with commercial software (GraphPad Prism 6; La Jolla, CA) using ANOVA with Fisher's LSD post hoc analysis. Values of $p<0.05$ were considered statistically significant.

CHAPTER IV

High glucose-induced apoptosis via the GAPDH/Siah1 pathway

A. High glucose induces Siah1 upregulation in human retinal pericytes

One of the challenges of studying chronic diseases like diabetes is trying to determine the ideal stimuli to model the disease *in vitro*. In order to address this challenge cell cultures are treated with disease relevant stimuli to mimic the environment present in patients with chronic diabetes. These stimuli are determined by examining the factors that are significantly elevated in diabetic patients when compared to non-diabetic controls. Even though the exact molecular mechanisms responsible for the onset and progression of diabetic retinopathy (DR) remains unknown, it is widely understood that chronic high glucose exposure during diabetes results in vascular damage [138]. Hence, primary human retinal pericytes (hRP) were cultured in high levels of D-glucose in order to model the hyperglycemic environment present in blood vessels during DR. D-glucose is a natural form of glucose, which is used by organisms as a primary energy source. In order to recreate normal blood sugar levels in cultured cells, hRPs were grown in 5.5mM D-glucose. This glucose concentration is roughly equivalent to 90mg/dl blood sugar level. In order to model high blood sugar levels, hRPs were cultured in 25mM D-glucose a concentration that is analogous to blood sugar levels of about 400mg/dl. It is important to note that patients with fasting blood sugar levels higher than 125mg/dl are considered diabetic [139]. Thus, a blood glucose concentration of 25mM D-is equivalent to severe diabetes.

After determining a relevant stimulus to model diabetic conditions in cells, hRPs were cultured in normal glucose (5mM D-glucose), osmotic control (25mM L-glucose) or

high glucose (25mM D-glucose) for 48hrs [140]. The use of L-glucose served as an important control since it has been previously shown that high glucose exposure causes osmolality changes in several cell types [141]. Therefore, throughout these studies L-glucose was used to control for any potential effects of osmotic stress resulting from stimulation with concentrated D-glucose. Unlike D-glucose, L-glucose cannot be phosphorylated by hexokinase, the first enzyme in the glycolysis pathway; therefore organisms cannot use L-glucose as a source of energy and has been shown to not cause diabetes like changes in the culture [142]. These biochemical properties of L-glucose make it an ideal osmotic control.

The first step in analyzing the involvement of the GAPDH/Siah1 pathway in human retinal pericyte cell death is to determine if Siah1 protein levels are elevated by disease relevant treatment. Under normal conditions Siah1 levels are constitutively active and can be found in low levels but under pathological conditions it has been shown that Siah1 protein and mRNA levels significantly increase [124]. After treatment with each stimulus, hRPs were lysed and total protein was collected from each sample. Siah1 total protein levels were analyzed using western blot analysis. Siah1 total protein increased 2-fold in cultures treated with high glucose (25mM D-glucose) compared to those treated with the osmotic control ($p=0.0136$). There was no significant difference between osmotic control and normal glucose-treated cells (**Figure 3**).

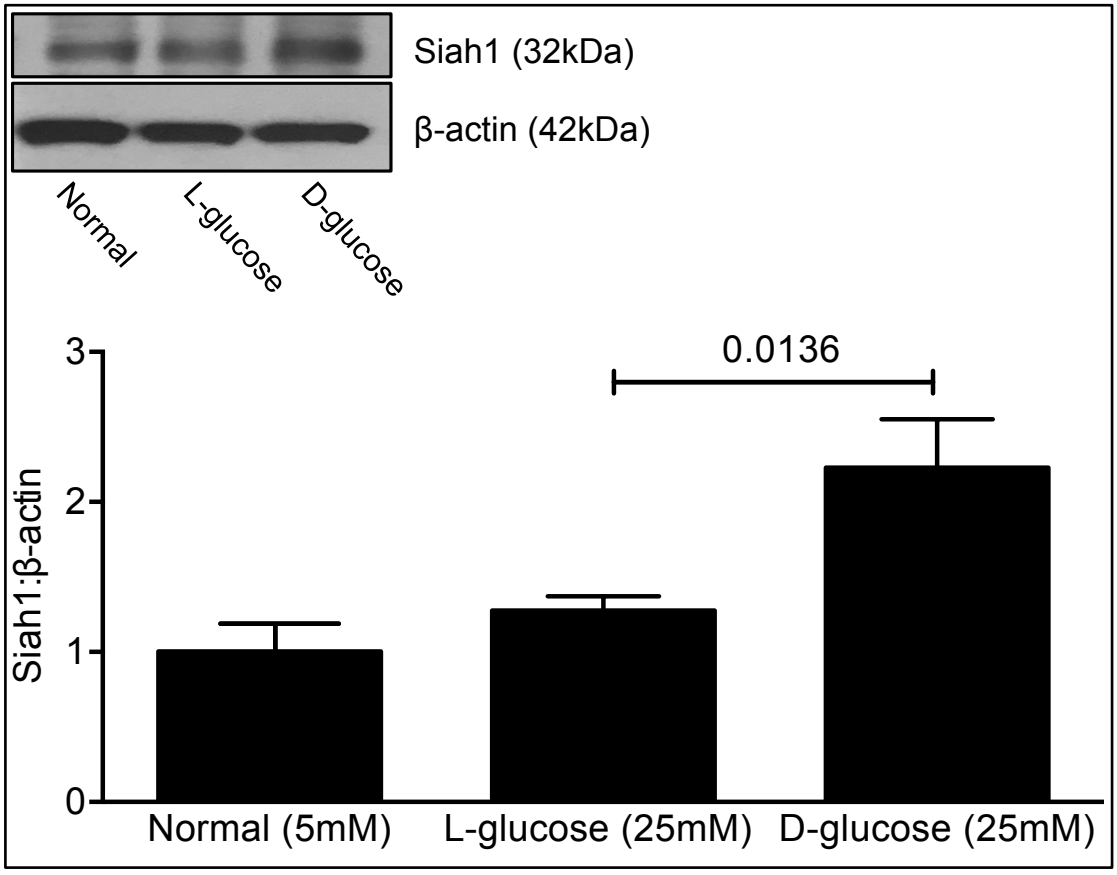


Figure 3. High glucose causes an upregulation of Siah1 total protein. hRP treated with high glucose (25mM D-glucose) for 48hrs have increased Siah1 total protein levels when compared to cells treated with either normal D-glucose (5mM) or L-glucose (25mM) (osmotic control) (**top**). Quantification of three independent experiments (**bottom**). β-actin serves as a marker for total protein loaded in each western blot. P=0.0136.

B. High glucose causes an increase in the association between GAPDH and Siah1

Association and binding of GAPDH and Siah1 is a crucial step that results in Siah1 stabilization and subsequent nuclear translocation of GAPDH. This interaction is important for the activation of the pathway in two ways. First, the association between GAPDH and Siah1 stabilizes the high turnover rate of Siah1. Under normal circumstances Siah1 functions as an E3 ligase, marking targets for proteasome mediated degradation. It is hypothesized that binding with GAPDH results in a decrease in Siah1s turnover rate allowing for prolonged E3 ligase activity [124]. Secondly, GAPDH/Siah1 association results in a protein complex that is then able to translocate into the nucleus resulting in down stream transcriptional events and leading to subsequent cell responses, such as cell death.

HRPs were cultured in the normal, L- or D- glucose for 48hrs, and samples were prepared for immunoprecipitation (IP) analysis in order to assay if hyperglycemic conditions would alter the association between GAPDH and Siah1. Immunoprecipitation assays were performed using anti-Siah1 antibody, followed by western blot (WB) analysis of the immuno-complexes with anti-GAPDH antibody. It is important to note that due to its high turnover rate, Siah1 is generally found in low abundance in hRPs. Therefore, precipitating Siah1 and analyzing the amount of GAPDH that is associated with Siah1 allows for better understanding of the association between Siah1 and GAPDH. Western blot analysis targeting GAPDH revealed a 1.5-fold increase in GAPDH/Siah1 association in high glucose-treated cells compared to those treated with the osmotic control ($p=0.0292$) (**Figure 4**).

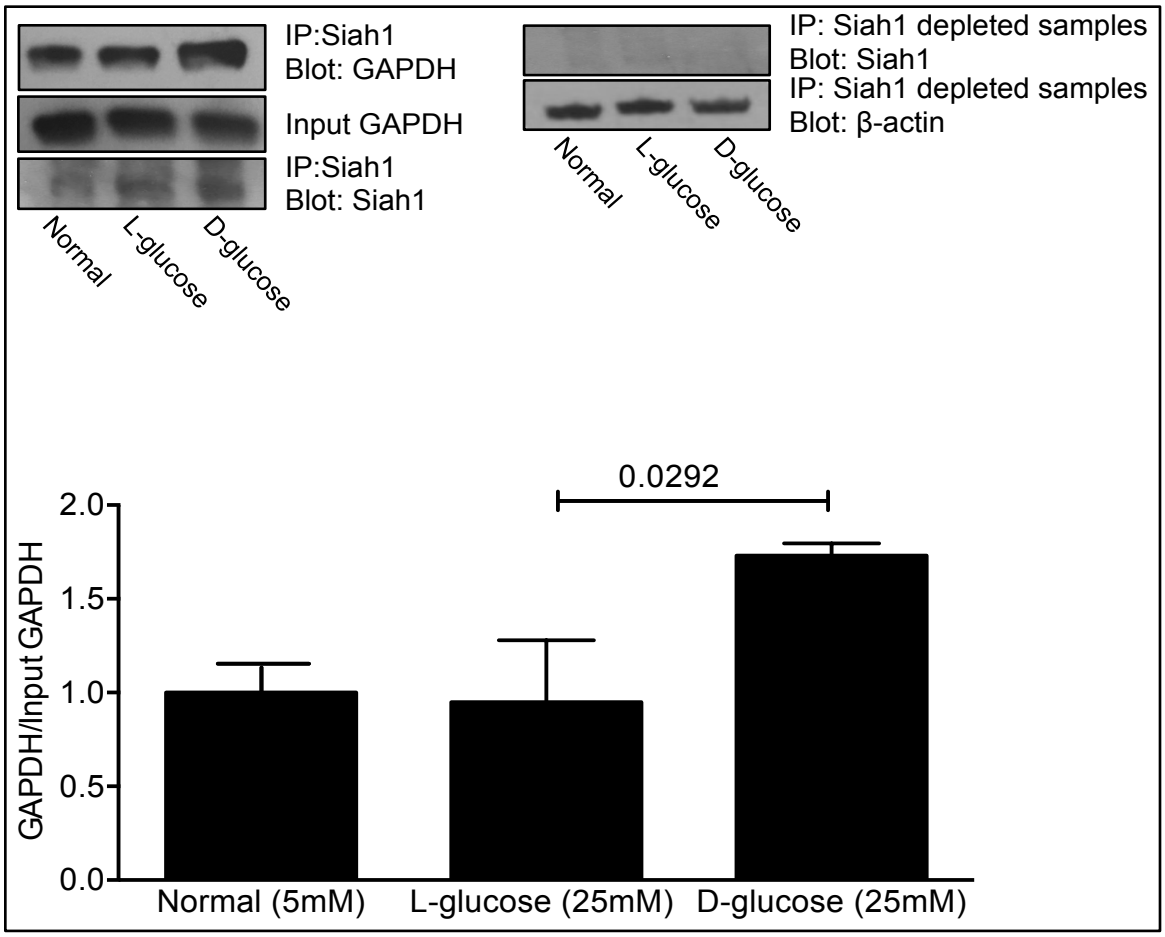


Figure 4. High glucose leads to an increase in the association between GAPDH and Siah1. Cells were treated with normal D-glucose (5mM), L-glucose (25mM) or high D-glucose (25mM) for 48hrs. hRP treated with high glucose have higher levels of GAPDH associated with Siah1 when compared to cells treated with either normal or L-glucose (**top**). Bottom graph demonstrates the quantification of three independent experiments. p=0.0292

C. High glucose causes GAPDH nuclear translocation

An essential step in the GAPDH/Siah1 pro-apoptotic pathway is nuclear translocation of GAPDH. For decades GAPDH has been recognized as a housekeeping gene, because it is believed that GAPDH's relative expression remains constant throughout most experimental manipulations [143]. Although this remains accurate for most cell types, in several cell types such as neuronal cells, retinal glial cells and retinal pericytes, diverse localization patterns of GAPDH lead to pathological outcomes such as cell death [124].

After 48hrs of treatment with normal glucose, osmotic control or high glucose, hRP lysates were prepared and separated into cytoplasmic and nuclear fractions. Each fraction was then subjected to GAPDH, MEK and Histone H3 western blot analysis. MEK and Histone H3 were used as control antigens to assess the purity of the cytoplasmic and nuclear fractions, respectively. High glucose treatment caused significant disappearance of cytosolic GAPDH and accumulation of nuclear GAPDH when compared to either normal glucose or osmotic control treatments ($p=0.0061$) (**Figure 5**). Immunocytochemical analysis was also performed under similar conditions to validate the nuclear and cytosolic western blot results. As expected, high glucose resulted in GAPDH nuclear translocation after 48hrs (**Figure 6**).

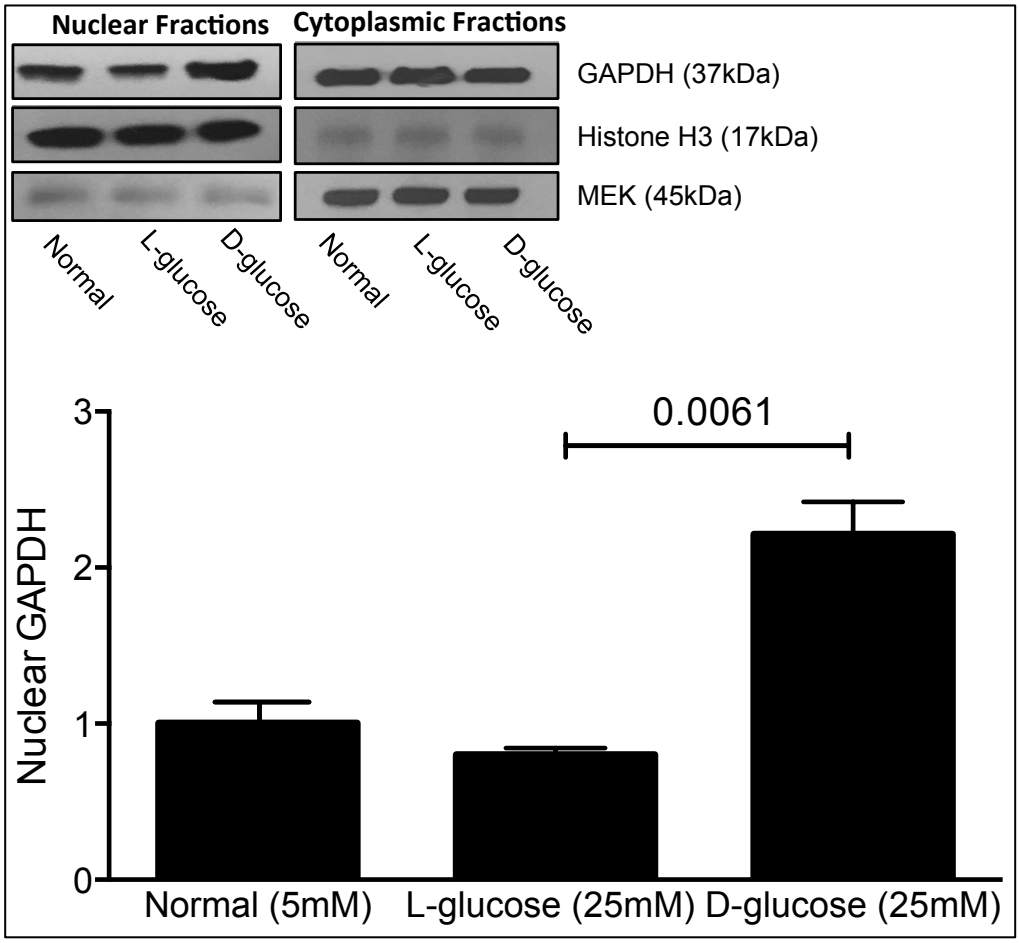


Figure 5. High glucose causes GAPDH nuclear translocation. Nuclear levels of GAPDH are significantly increased in hRP treated with high D-glucose (25mM) for 48hrs when compared to cells treated with normal (5mM) or L-glucose (25mM) (p=0.0061) (**top**). Bar graph demonstrates quantification of nuclear GAPDH from three independent western blot experiments (**bottom**).

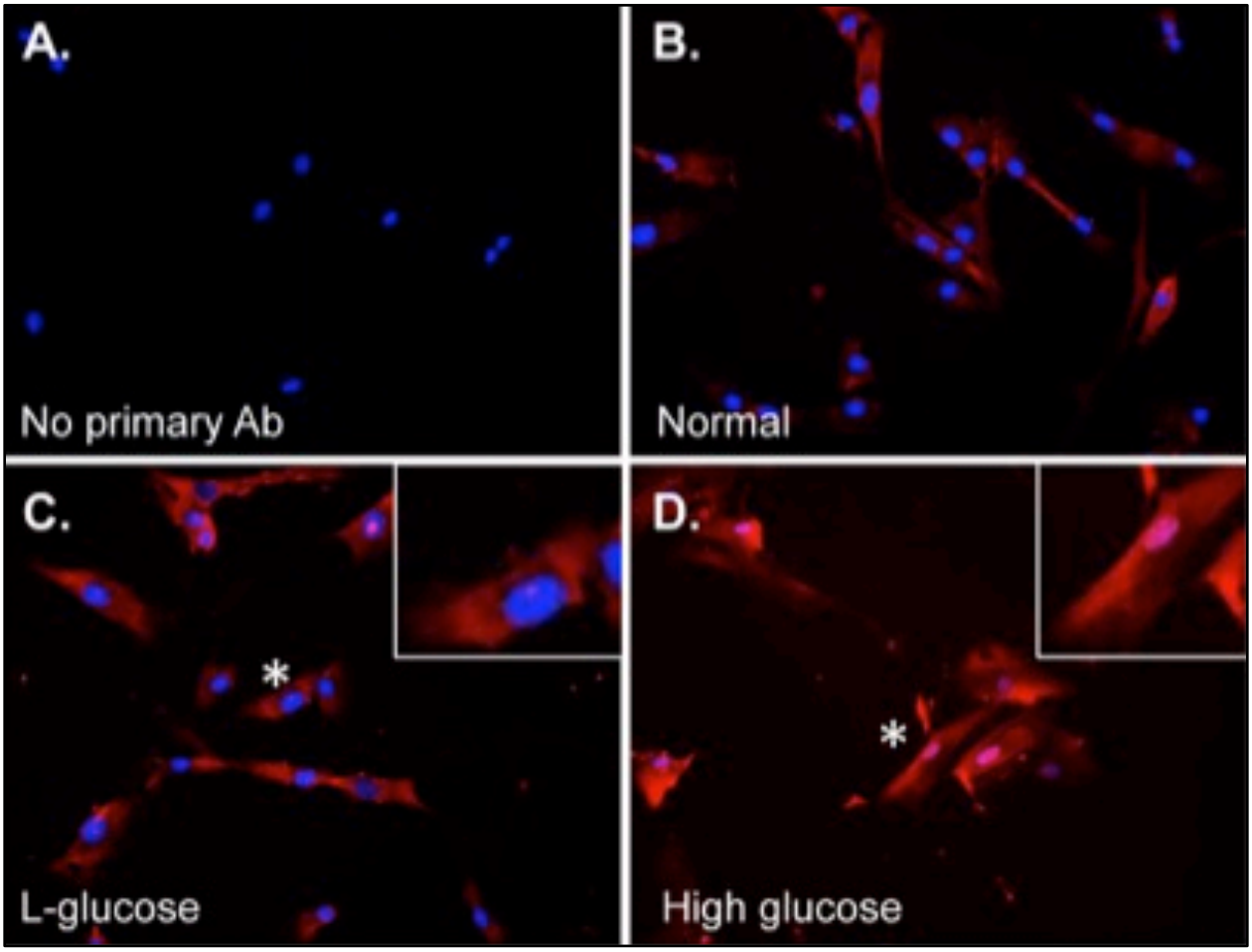


Figure 6. Immunocytochemical analysis of GAPDH nuclear translocation. HRP were treated with **A**) no primary control (**B**) normal glucose (5mM), (**C**) L-glucose (25mM) and (**D**) high glucose (25mM). GAPDH is shown in red, while DAPI- stained cell nuclei are shown in blue.

D. High glucose causes hRP apoptosis

In order to examine the effects of high glucose exposure on hRP survival, primary cultures of hRPs were incubated in normal glucose, L-glucose or high glucose for 48hrs-72hrs. Cell death is evident after 48hrs of high glucose treatment and it is significantly increased after 72hrs. Treatment with 25mM D-glucose for 72hrs resulted in a 3-fold increase in caspase-3-enzymatic activity ($p<0.0001$). It is well documented that

activation of pro-apoptotic caspases, such as caspase 3, leads to downstream activation of pro-apoptotic signaling events, ultimately resulting in apoptosis [144]. Similarly, high glucose exposure also caused a significant increase in Annexin V levels, another measure of apoptosis-specific cell death ($p<0.0001$) (**Figure 7**). Taken together the data presented thus far suggests that in human retinal pericytes high glucose causes GAPDH/Siah1 association that results in nuclear translocation of GAPDH and ultimately results in hRP apoptosis.

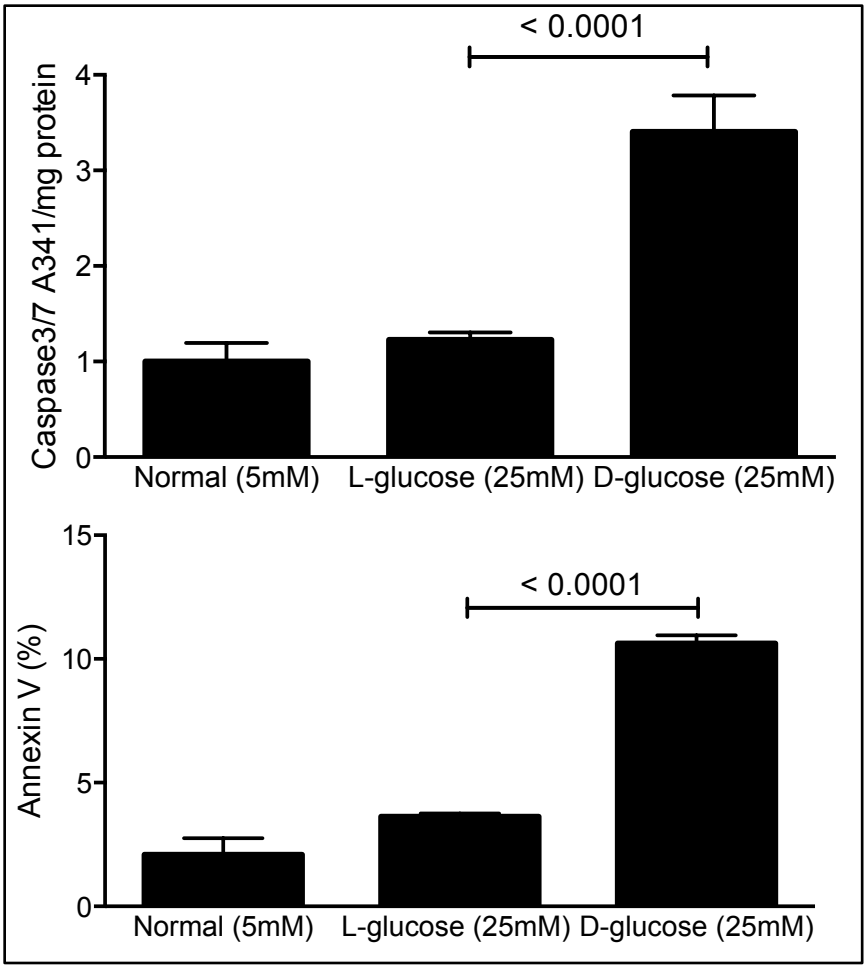


Figure 7. High glucose causes human retinal pericyte apoptosis. Cells were treated with normal glucose (5mM), L-glucose (25mM) or high glucose (25mM) for 48hrs. Caspase-3 enzymatic activity and Annexin V levels were measured as markers for apoptosis. High glucose significantly upregulated both caspase-3 enzymatic activity (**top**) and Annexin V levels (**bottom**) when compared to normal or L-glucose.

Since immunoprecipitation studies showed that high glucose results in a significant increase in GAPDH and Siah1 association, it would be valuable to elucidate the events occurring upstream of this interaction. Further understanding of this pro-apoptotic pathway may allow for the development of inhibitory therapies to prevent high glucose-induced cell death. According to the proposed mechanism of action of the GAPDH/Siah1 pathway, high glucose causes an increase in nitric oxide synthase (NOS) activity, which results in elevated intracellular levels of nitric oxide (NO) (**Figure 2**). NO plays several important biological roles. For instance, NO has been shown to modulate cell communication, immune defense against pathogens, vasodilation, as well as to modulate platelet aggregation and leukocyte endothelial interactions [145]. NO modulates these and other key biological process by either activating or blocking gene transcription. Interestingly, serum NO levels are significantly elevated in patients with diabetic retinopathy when compared to normal matched controls [146]. NOS synthesizes NO from L-arginine as a free radical [147]. Since NO is highly unstable, measuring NOS activity serves as a way to measure intracellular levels of NO. Using a NOS activity kit, hRPs were cultured in control, L- or D-glucose conditions. After 48hrs cells were collected and NOS activity was measured. In biological systems the final products of NO are nitrite (NO^{2-}) and nitrates (NO^{3-}). Since the total proportions of these products is highly variable, accounting for both forms allows for the measurement of total NO produced in each control or experimental condition. Using this approach, hRPs cultured in D-glucose showed a modest but significant increase in NO levels when compared to cells cultured in normal or L-glucose conditions ($p=0.0037$) (**Figure 8**).

It is also hypothesized that increases in GAPDH S-nitrosylation levels occur as a function of increased levels of intracellular NO. S-nitrosylation is a form of post-translational modification that facilitates the binding between GAPDH and Siah1 [148]. This post-translational modification has been shown to modulate several key cellular events such as proliferation, differentiation and apoptosis [149]. The Pierce S-nitrosylation western blot kit was used to measure S-nitrosylated total hRP protein levels. This assay relies on the cellular events that occur during S-nitrosylation. During this post-translational modification NO radicals react with cysteine thiols resulting in S-nitrosylated proteins (**Appendix C**). In turn these proteins, like GAPDH, have significantly altered activity. **Figure 9** demonstrates the amount of S-nitrosylated proteins present in hRPs cultured in normal, L- or D- glucose for 48hrs. In this assay ascorbate was used to selectively reduce S-nitrocysteines, allowing for the subsequent labeling with the idoTMTzero reagent. This reagent is designed to specifically label modified cysteine adducts. Thus, cells treated with purified water instead of ascorbate serve as a negative control in this assay.

In summary, high glucose causes an increase in the association between GAPDH and Siah1 by elevating levels of NO-induced GAPDH S-nitrosylation. This post-translationally altered form of GAPDH alters affinity for, and facilitates interaction with, Siah1, which in turn activates the GAPDH/Siah1 pro-apoptotic pathway. All of these events ultimately result in high glucose-induced hRP apoptosis.

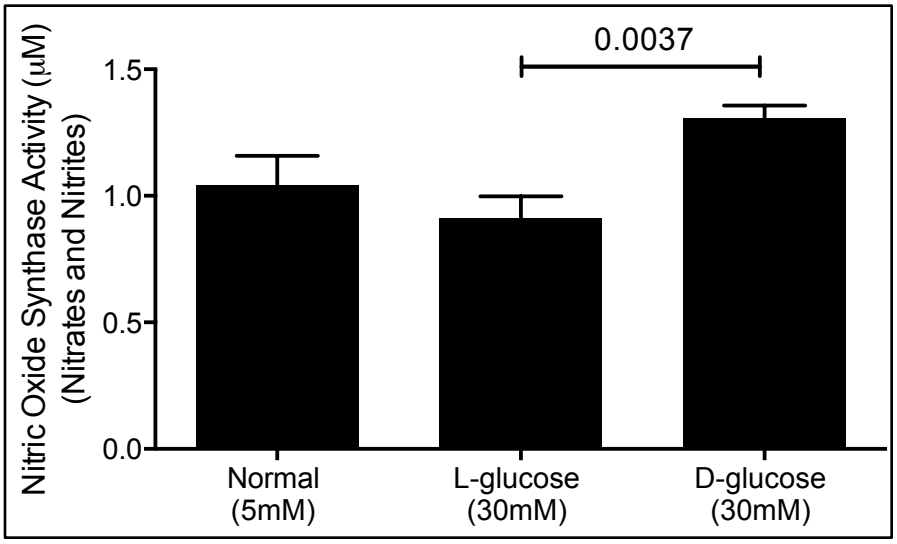


Figure 8. High glucoses causes an increase in nitric oxide synthase (NOS). HRPs were treated with low glucose (5mM), 25mM L- or D-glucose for 48hrs. NOS activity was measured using the Calbiochem NOS colorimetric kit. Graph represents total nitrite (NO^{2-}) and nitrate (NO^{3-}) levels.

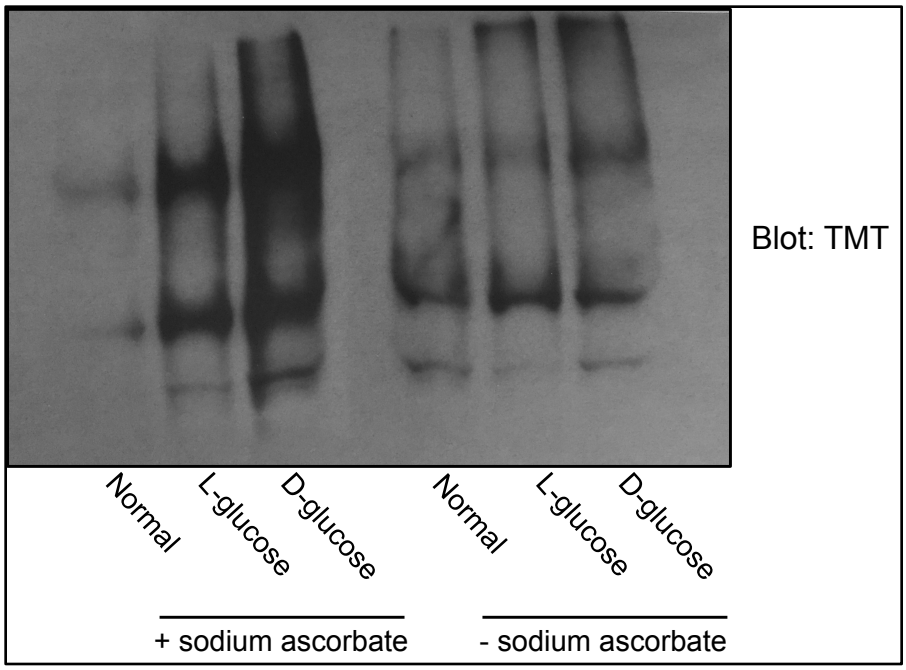


Figure 9. Western blot analysis of S-nitrosylated proteins. Using the Pierce S-nitrosylation western blot kit, S-nitrosocysteines are selectively reduced with ascorbate for labeling with iodoTMTzero reagent. The anti-TMT antibody was used for western blot detection of the TMT-labeled proteins. Samples treated with no ascorbate serve as negative controls.

CHAPTER V

Inhibition of the GAPDH/Siah1 pathway prevents high glucose-induced hRP apoptosis

A. Pharmacologic inhibition of the GAPDH/Siah1 pathway with R-Deprenyl

Our evidence suggests that high glucose results in Siah1 total protein upregulation followed by an increased association between GAPDH and Siah1. This association in turn leads to GAPDH nuclear translocation and cell death. After determining that the GAPDH/Siah1 pathway was involved in high glucose-induced cell death, it became apparent that inhibition of the pathway may render a method of suppressing pericyte apoptosis and progression of diabetic retinopathy (DR). In order to inhibit the GAPDH/Siah1 pathway, three tools were employed. The first is a selective and irreversible inhibitor of the B-isoform of monoamine oxidase (MAO-B) enzyme known as R-deprenyl (selegiline) [150]. This drug is currently used to treat early stages of Parkinson's disease, depression and dementia [151]. Additionally, R-deprenyl has been shown to be neuroprotective and anti-apoptotic in concentrations too low to affect MAO-B activities, although the mechanisms of these activities remain unconfirmed [152]. Another group studying GAPDH/Siah1 interactions in retinal glial cells also has shown that R-deprenyl prevents GAPDH nuclear translocation and cell death [124].

Human retinal pericytes were cultured in normal glucose (5mM), L-glucose (25mM), D-glucose (25mM) and/or R-deprenyl (1nM). This concentration of R-deprenyl was selected based on published work done in retinal glial cells that successfully demonstrated deprenyl's capability to block high glucose-induced GAPDH nuclear translocation [134]. As previously demonstrated, hRPs treated with D-glucose showed significant increased levels of Siah1 total protein. Cells treated with D-glucose in

addition to R-deprenyl showed significantly decreased levels of Siah1 total protein when compared to cells treated with D-glucose alone (p=0.0004) (**Figure 10**). These results suggest that R-deprenyl prevented GAPDH/Siah1 association, since this association is known to stabilize Siah1 total protein.

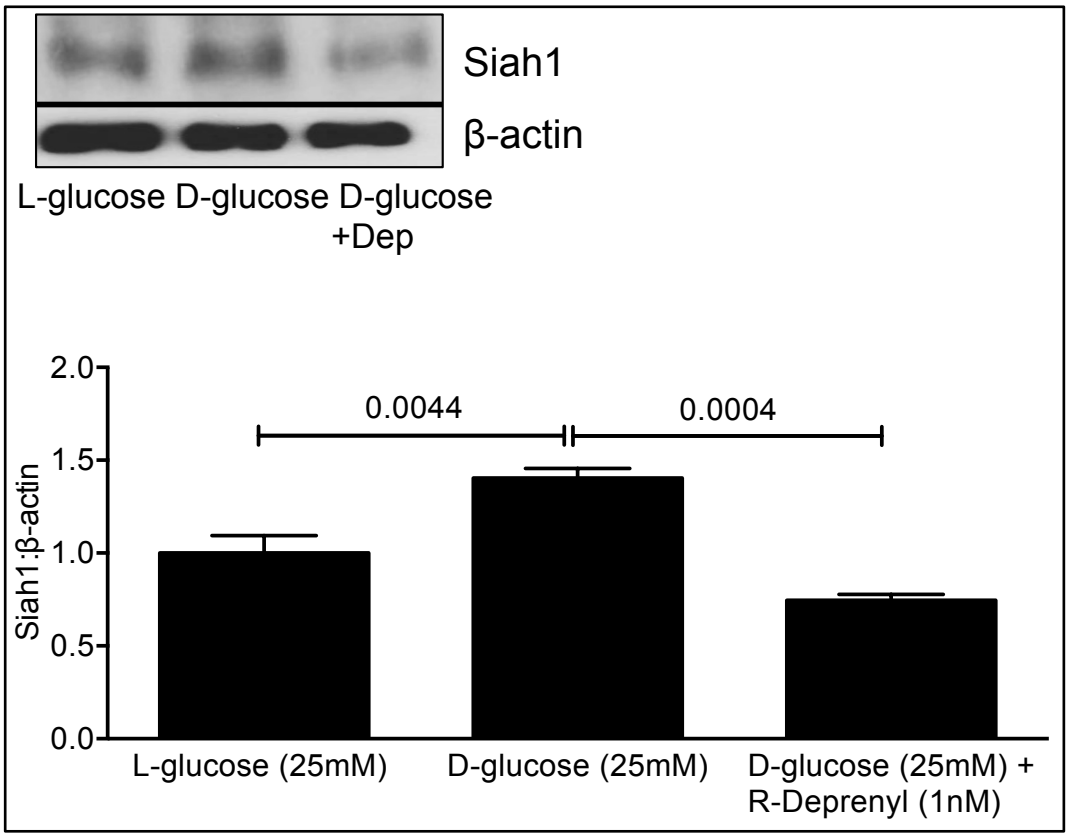


Figure 10. Deprenyl inhibits high glucose-induced Siah1 total upregulation. HRPs were treated with L-glucose (25mM), high D-glucose (25mM) for 48hrs. Cells treated with R-Deprenyl (1nM) in the presence of high glucose show significantly decreased levels of Siah1 total protein (p=0.0004) when compared to high glucose treated pericytes (p=0.0044). β-actin was used as a loading control for total protein concentration. Quantification of three independent experiments is demonstrated in the bar graph.

In order to examine the effect of R-deprenyl on GAPDH/Siah1 association, GAPDH immunoprecipitation studies were conducted in hRPs cultured in D-glucose in the absence or presence of R-deprenyl. Immunoprecipitation analysis was conducted as stated in *Chapter 3 Section B*. As expected, hRPs cultured in high glucose over 48hrs showed significantly elevated levels of GAPDH/Siah1 association when compared to cells treated with high glucose plus R-deprenyl ($p=0.0009$) (**Figure 11**). Although the mechanism in which R-deprenyl blocks GAPDH/Siah1 association remains unknown, several groups have hypothesized that since R-deprenyl inhibits nitric oxide production this decrease in NO causes a decrease in the amount of S-nitrosylated GAPDH [119, 120]. Furthermore, inhibition of NO production by using L-NAME was able to abolish GAPDH/Siah1 binding and nuclear translation of GAPDH [119]. Because binding to Siah1 depends on formation of SNO-GAPDH, a decrease in the amount of SNO-GAPDH is expected to result in a decrease in GAPDH/Siah1 interaction. Additionally, administration of R-deprenyl (1nM) blocks high glucose-induced GAPDH nuclear translocation. These results are demonstrated by immunocythochemistry analysis (**Figure 12**). Lastly, treatment with R-deprenyl blocked high glucose-induced caspase-3 enzymatic activity. These results indicate that inhibition of GAPDH/Siah1 association prevents high glucose-induced apoptosis ($p<0.0001$) (**Figure 13**).

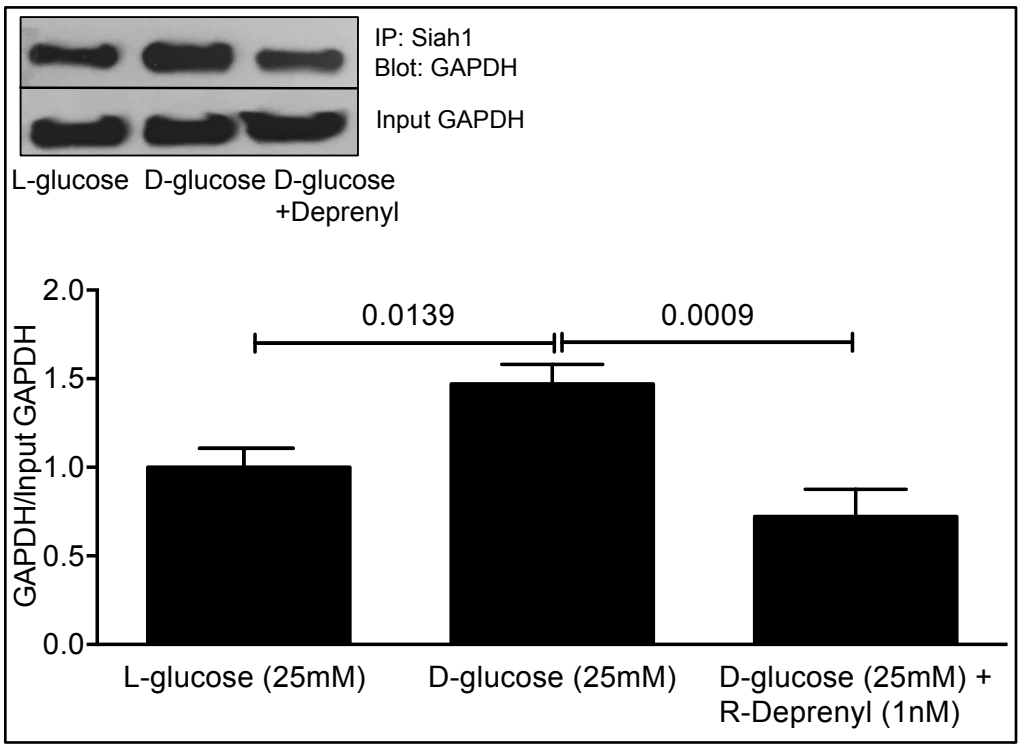


Figure 11. Deprenyl inhibits high glucose-induced GAPDH/Siah1 association. HRPs were treated with L-glucose (25mM), high D-glucose (25mM) or high glucose plus R-Deprenyl (1nM). Cells treated with R-Depreny in the presence of high glucose show significantly decreased levels of GAPDH/Siah1 association (p=0.0009) when compared to high glucose treated pericytes (p=0.0139) as determined by immunoprecipitation (**top**). Input GAPDH was used to determine total GAPDH present in each sample. Bar graph demonstrates the quantification of three independent experiments (**bottom**).

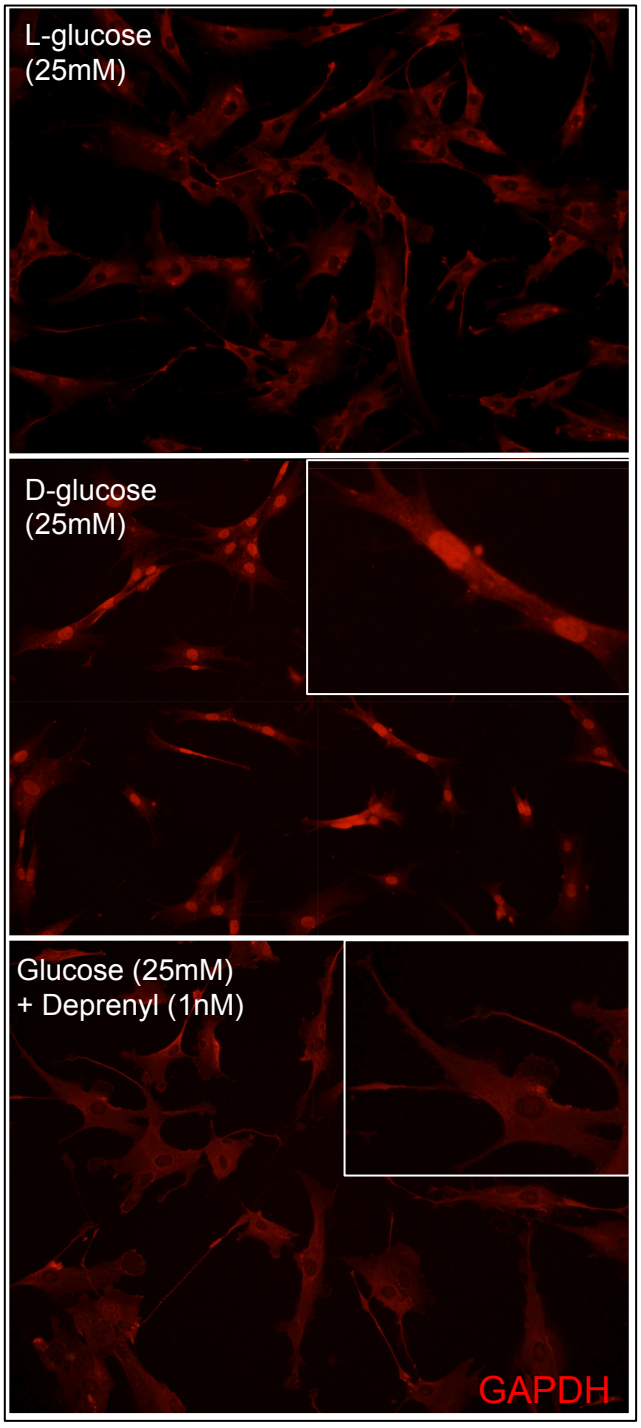


Figure 12. Deprenyl inhibits high glucose-induced GAPDH nuclear translocation. HRPs were treated with L-glucose (25mM), high D-glucose (25mM) or high glucose plus R-Deprenyl (1nM). Cells treated with high glucose show significantly increased levels of GAPDH nuclear translocation (**middle panel**) when compared to cells treated with high glucose in the presence of R-Deprenyl (**bottom panel**). GAPDH is shown in red.

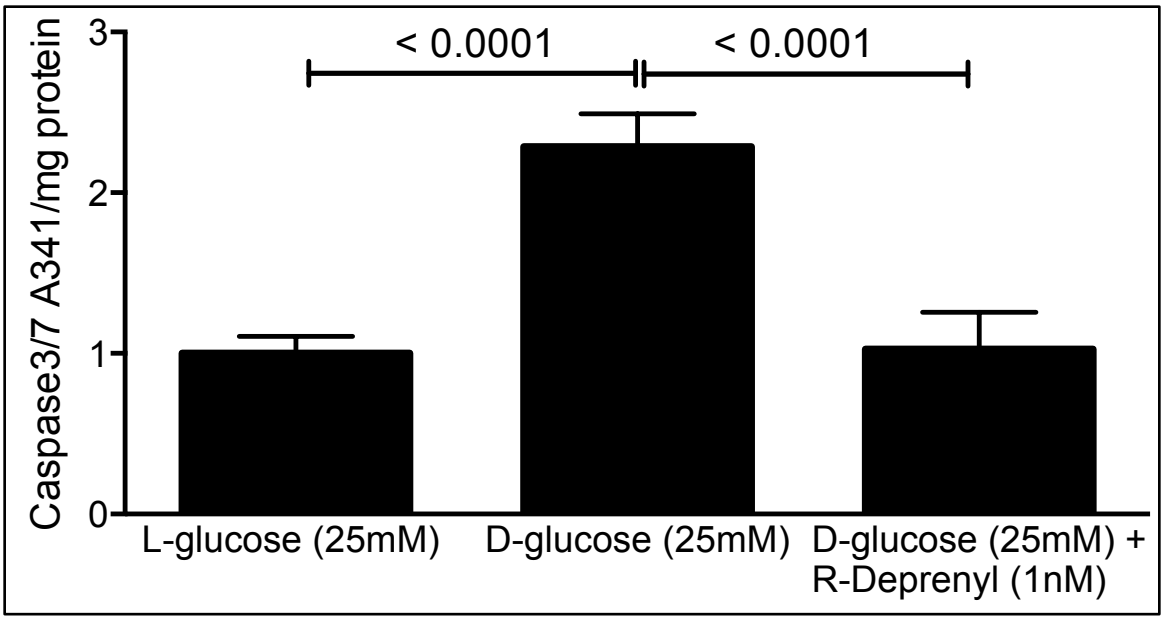


Figure 13. R-Deprenyl prevents high glucose-induced human retinal pericyte apoptosis. Cells were treated with normal glucose (5mM), L-glucose (25mM) or high glucose (25mM) for 48hrs. Cells treated with R-Deprenyl for 24hrs show significantly decreased levels of caspase-3 enzymatic activity when compared to cells treated with high glucose alone. $P<0.0001$.

B. Inhibition of the GAPDH/Siah1 pathway with Siah1 siRNA

Although it appears that R-deprenyl successfully inhibits high glucose-induced hRP apoptosis in a GAPDH/Siah1 specific manner, the mechanism of action of R-deprenyl in this context still remains under active investigation. Therefore, using a more specific form of inhibition, such as siRNA against Siah1, allows for defined inhibition of the GAPDH/Siah1 pathway. In order to inhibit Siah1 message levels, a pool of four non-overlapping siRNA sequences directed at Siah1 were used (**Appendix B**). In these studies a negative control oligomer sequence was used in order to account for the effect of the transfection protocol on hRPs. After confirming Siah1 knockdown (**Figure 14**), hRPs were cultured in L-glucose, D-glucose and D-glucose plus Siah1 siRNA. In addition to these conditions, cells were also cultured in L- or D-glucose in the presence of negative control siRNA. As expected, hRPs treated with negative control siRNA plus D-glucose show increased levels of GAPDH/Siah1 association. This high glucose-induced association is significantly inhibited in hRPs treated with Siah1 siRNA (p=0.0461) (**Figure 15**).

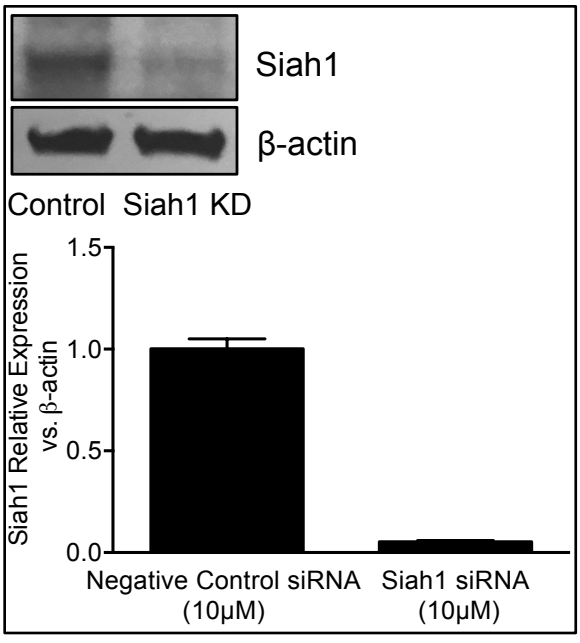


Figure 14. Siah1 knock-down (KD) efficiency. **A**) Siah1 expression and (**B**) protein levels are significantly reduced with 10μM Siah1 directed siRNA oligomers. Expression levels are measured by RT-PCR and protein levels are measured by western blot analysis.

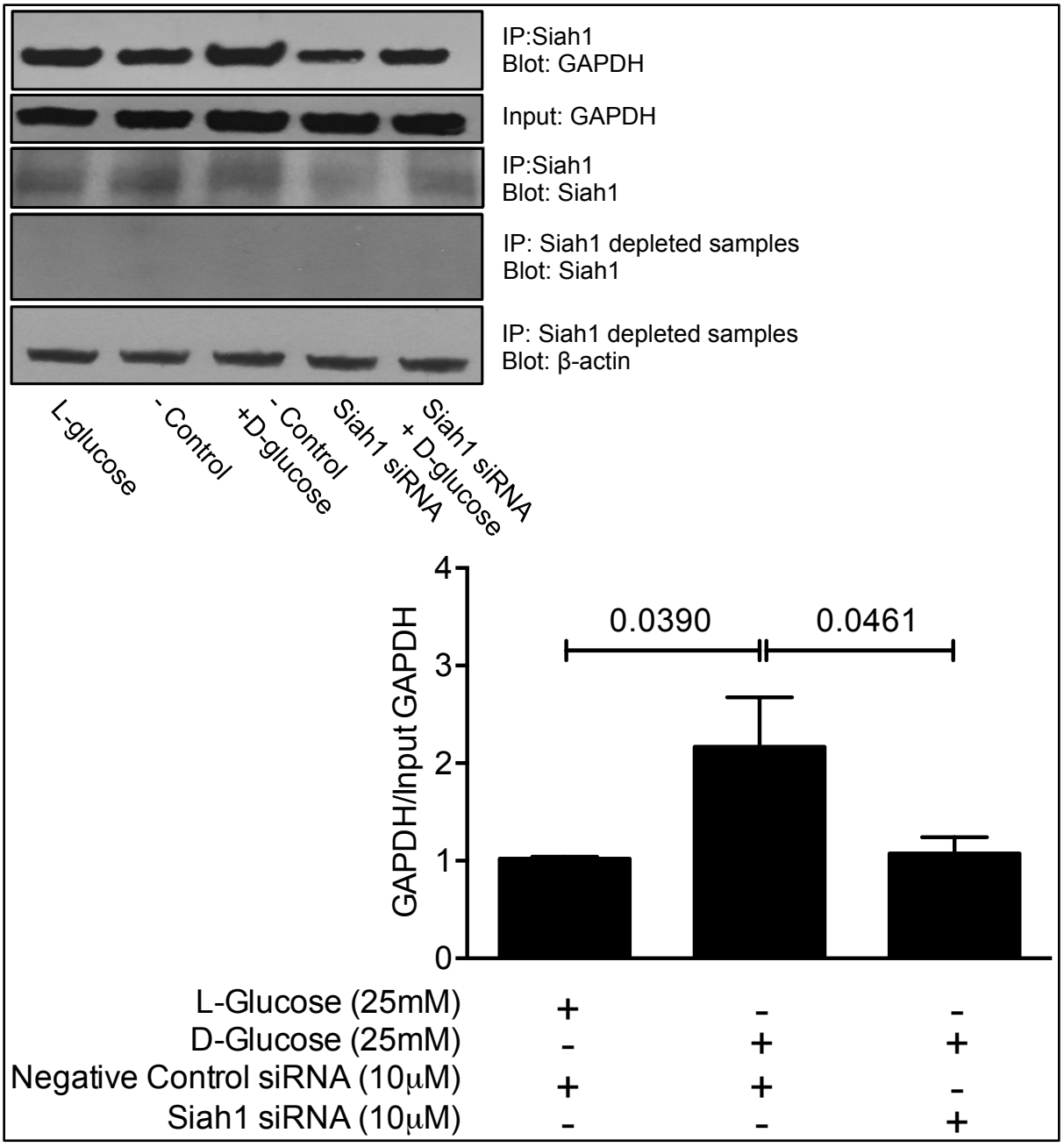


Figure 15. Inhibition of the GAPDH/Siah1 pathway with Siah1 siRNA prevents high glucose-induced GAPDH/Siah1 association. Inhibition of Siah1 with Siah1 siRNA (10μM) inhibits high glucose-induced GAPDH/Siah1 association. Quantification of three independent experiments is demonstrated in the bar graph.

More importantly, nuclear fractions isolated from hRPs treated with Siah1 siRNA in the presence of high glucose treatment show decreased levels of GAPDH/Siah1 association when compared to cells treated with negative control siRNA plus high glucose. These results suggest that the GAPDH/Siah1 association is not only occurring in the cytosolic portion of the cell but the pathway is also active in the nuclear portion as well (**Figure 16**).

Also, in accordance with the proposed GAPDH/Siah1 mechanism of action, inhibition of Siah1 with Siah1 directed siRNA prevented high glucose-induced GAPDH nuclear translocation as demonstrated in (p=0.0227) **Figure 17**. Lastly, Siah1 siRNA treatment blocked high glucose-induced caspase-3 enzymatic activity suggesting that the E3 ligase, Siah1, is a crucial and necessary player in the GAPDH/Siah1 pro-apoptotic pathway (p=0.0049) (**Figure 18**).

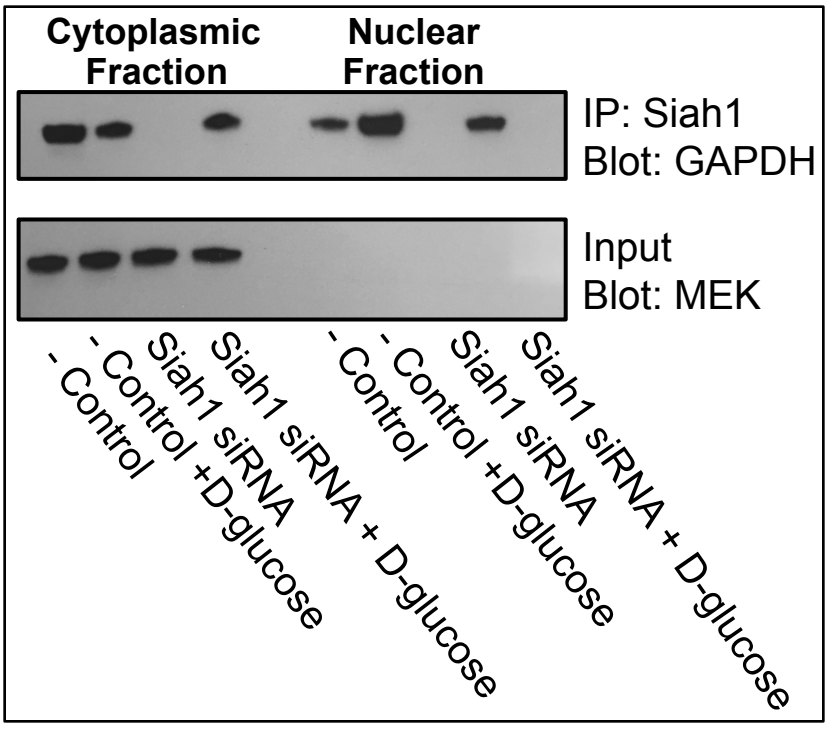


Figure 16. Inhibition of the GAPDH/Siah1 pathway with Siah1 siRNA in hRP nuclear fractions. Nuclear fractions isolated from HRPs treated with negative control or Siah1 siRNA (10μM) in the presence or absence of D-glucose (25mM). Siah1 siRNA inhibits high glucose-induced GAPDH/Siah1 association in hRP nuclear fractions.

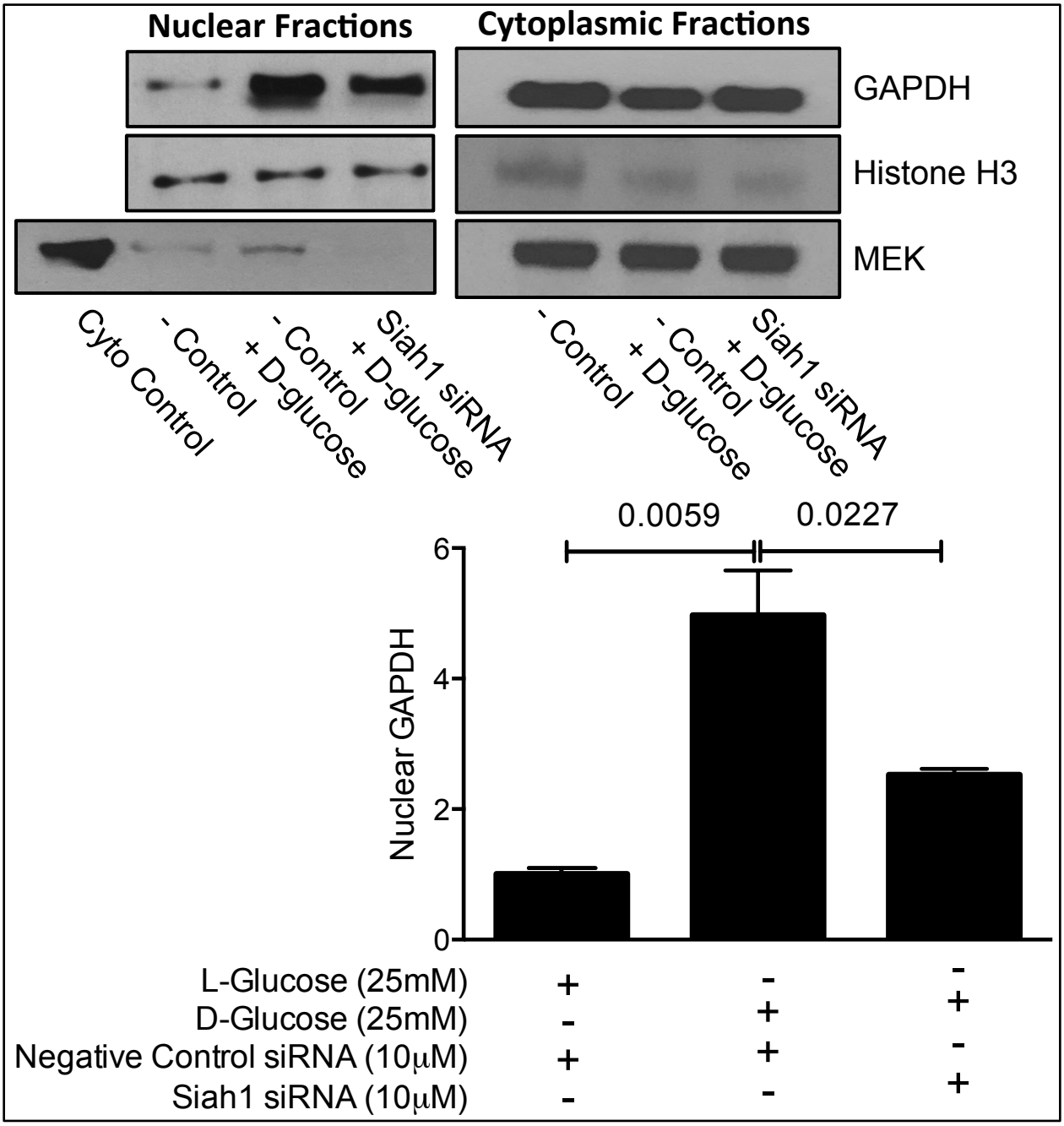


Figure 17. Inhibition of the GAPDH/Siah1 pathway with Siah1 siRNA prevents high glucose-induced GAPDH nuclear translocation. Siah1 siRNA also inhibits GAPDH/Siah1 association in HRP nuclear fractions. Histone H3 and MEK are used for positive and negative control, respectively, for purity of nuclear fraction isolations.

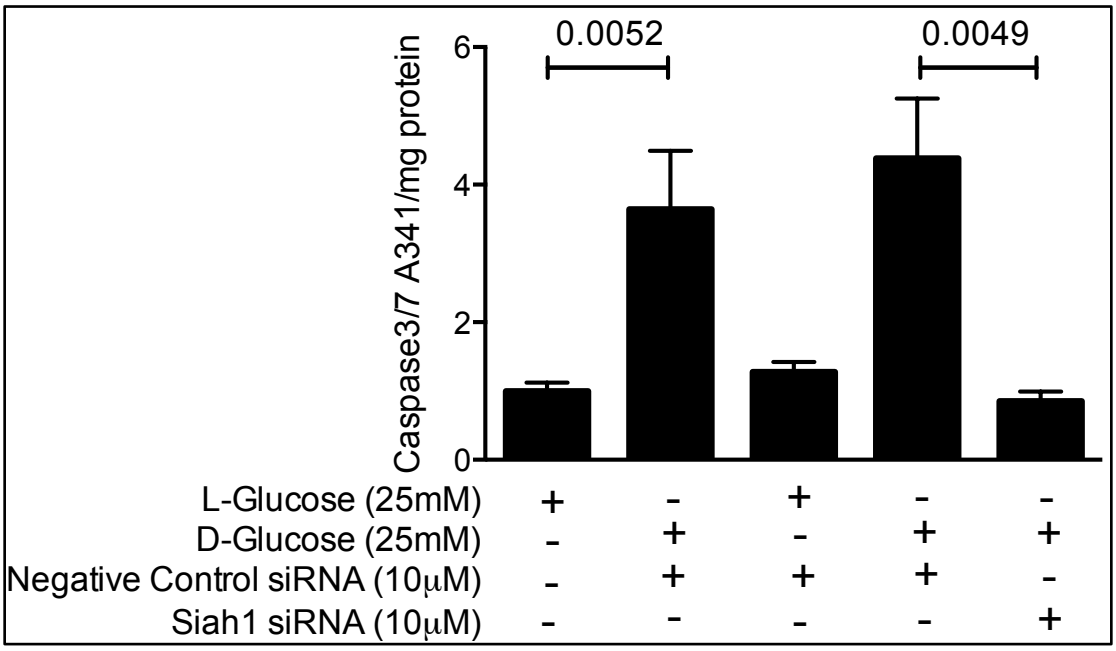


Figure 18. Siah1 siRNA inhibits high glucose-induced caspase-3 enzymatic activity. Cells were treated with L-glucose (25mM), D-glucose (25mM), negative control siRNA (10μM) or Siah1 siRNA (10μM). High glucose resulted in an increase in caspsae-3 enzymatic activity (P=0.0052) and this increase was significantly reduced in the presence of Siah1 siRNA (P=0.0049).

C. Inhibition of the GAPDH/Siah1 pathway with GAPDH/Siah1 blocking peptides

Lastly, in hopes of exclusively targeting the GAPDH/Siah1 pathway, TAT FLAG peptides were designed in collaboration with the Jayagapol Laboratory at Vanderbilt University to competitively block GAPDH and Siah1 binding. These peptides are transactivator of transcription (TAT) peptides (GRKKRRQRRRPQ), which are known as cell penetrating peptides. Although there are several methods of cargo delivery by these peptides, the most common and well studied is endocytosis-mediated translocation with the cargo being delivered into endosomes. Three peptides were used in this project. The first is referred to as the GAPDH peptide and it is designed to block the GAPDH binding site on Siah1. The second peptide is referred to as the Siah1 peptide and it blocks the Siah1 binding site on GAPDH. The third peptide is a control peptide that lacks specificity for either binding site and serves as a control for positive cell entry. Further peptide sequences and functions can be found in **Table 1**.

This FLAG tag is a polypeptide protein tag with sequence DYKDDDDK that can be used to identify peptide cell entry by anti-FLAG immunocytochemistry (**Figure 19A)** or immunoprecipitation (**Figure 19B)** analysis. It is important to note that cell viability assays was also performed using these peptides and they are not cytotoxic to hRPs (**Figure 19C)**.

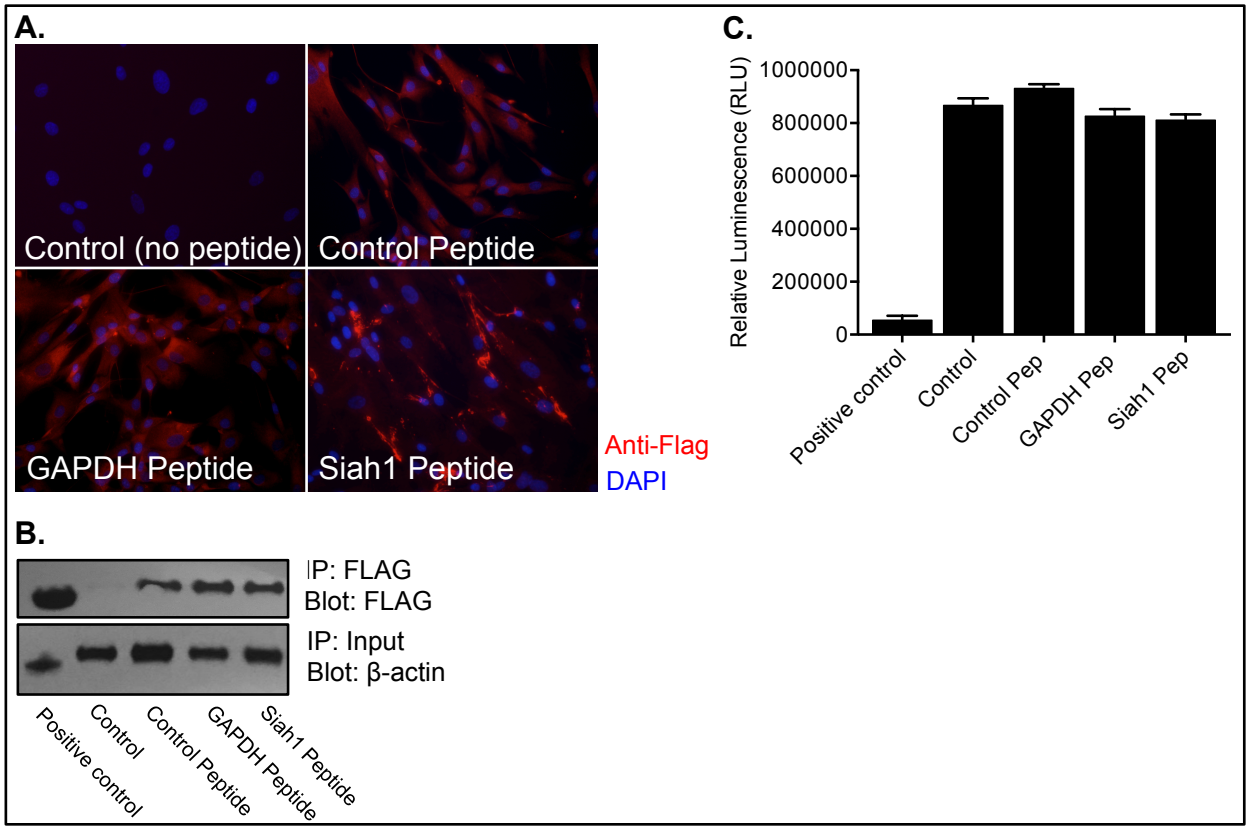


Figure 19. TAT-FLAG Peptide Identification. A) Immunocytochemistry analysis of anti- FLAG (red) staining in human retinal pericytes (hRP). Top left panel demonstrates hRPs cultured in control medium with no peptide treatment. This condition serves as a measure of background FLAG fluorescence. All four panels are stained with anti-Flag antibody. Nuclei are stained in blue with DAPI. Western blot analysis of anti-FLAG immunoprecipitates. FLAG-BAP Fusion Protein is used as a positive control to confirm the functional integrity of anti-FLAG monoclonal antibody (**B**). Cell viability assay of hRPs treated with corresponding peptide. Cells were treated with 70% methanol for 30mins as a positive control (**C**).

Treating hRPs with 1μM GAPDH, Siah1 or a combination of both peptides, inhibited high glucose-induced GAPDH/Siah1 association. This association, which is hypothesized to be a crucial step in the GAPDH/siah1 pro-apoptotic pathway, led to GAPDH nuclear translocation (GAPDH pep p=0.0142, Siah1 pep p=0.0221, GAPDH+Siah1 pep p=0.0100) (**Figure 20**). Selectively inhibiting GAPDH and Siah1 binding also inhibited high glucose-induced GAPDH nuclear translocation (**Figure 21**). Most importantly, each peptide was administered to pericytes in the presence or absence of high glucose. As previously demonstrated in earlier sections, pericytes cultured in high glucose demonstrated a significant increase in caspase-3 enzymatic activity. This increase in caspase-3 activity can be restored to baseline levels when GAPDH or Siah1 peptides are administered two hours before high glucose treatment (GAPDH pep p=0.0077, Siah1 pep p=0.0528) (**Figure 22**). These results suggest that treatment with R-deprenyl, can serve an anti-apoptotic treatment to block high glucose-induced pericyte loss.

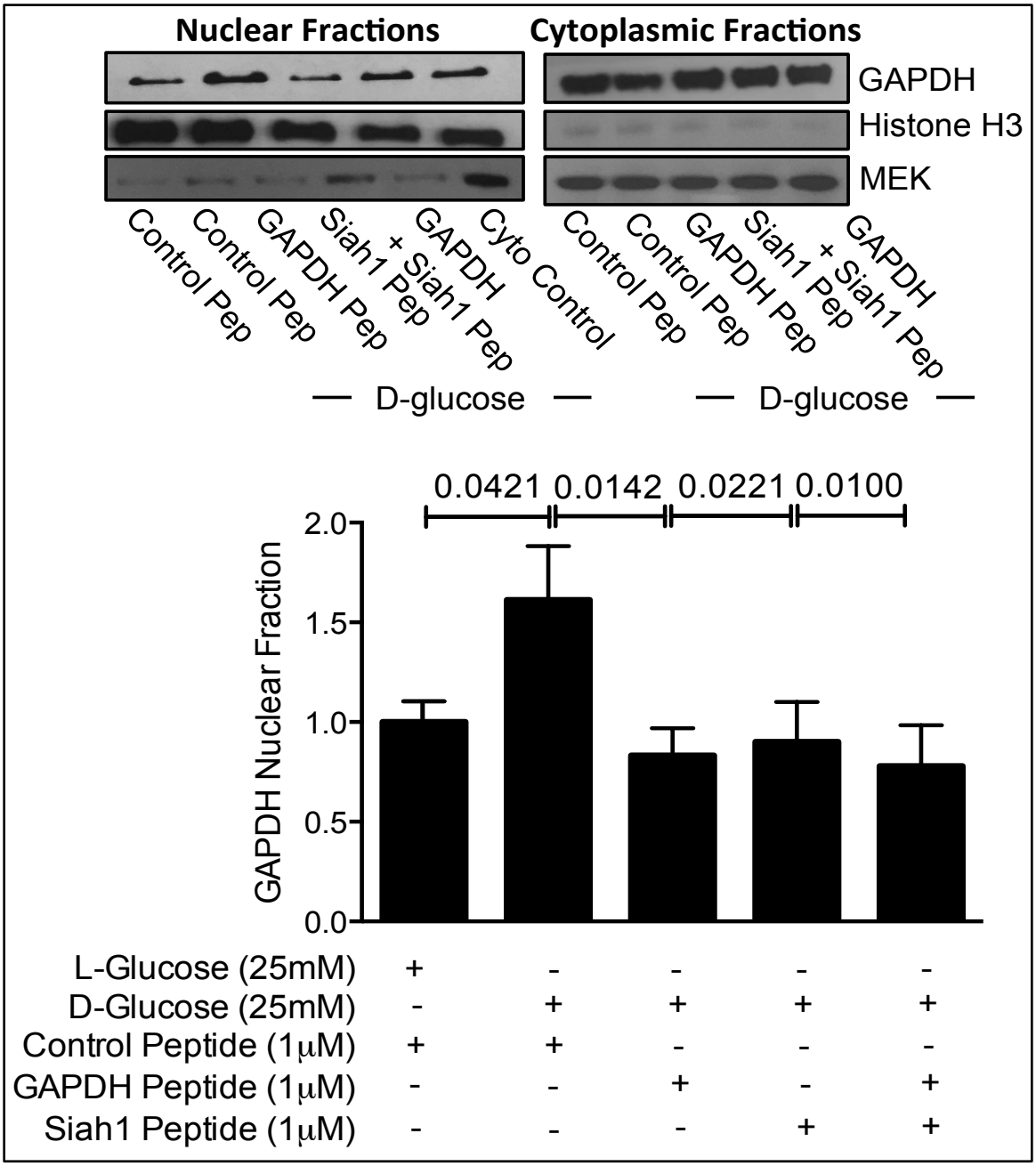


Figure 20. GAPDH/Siah1 blocking peptides block high glucose-induced GAPDH/Siah1 association. After a two-hour incubation with either control peptide (1μM), GAPDH peptide (1μM) or Siah1 peptide (1μM) hRPs were treated with D-glucose. Cells treated with high D-glucose (25mM) demonstrate a significant increase in association between GAPDH/Siah1. Blocking either the GAPDH binding site on Siah1 (p=0.0194), the Siah1 binding site on GAPDH (p=0.0066) or both binding sites (p=0.0146) inhibits this high glucose-induced association.

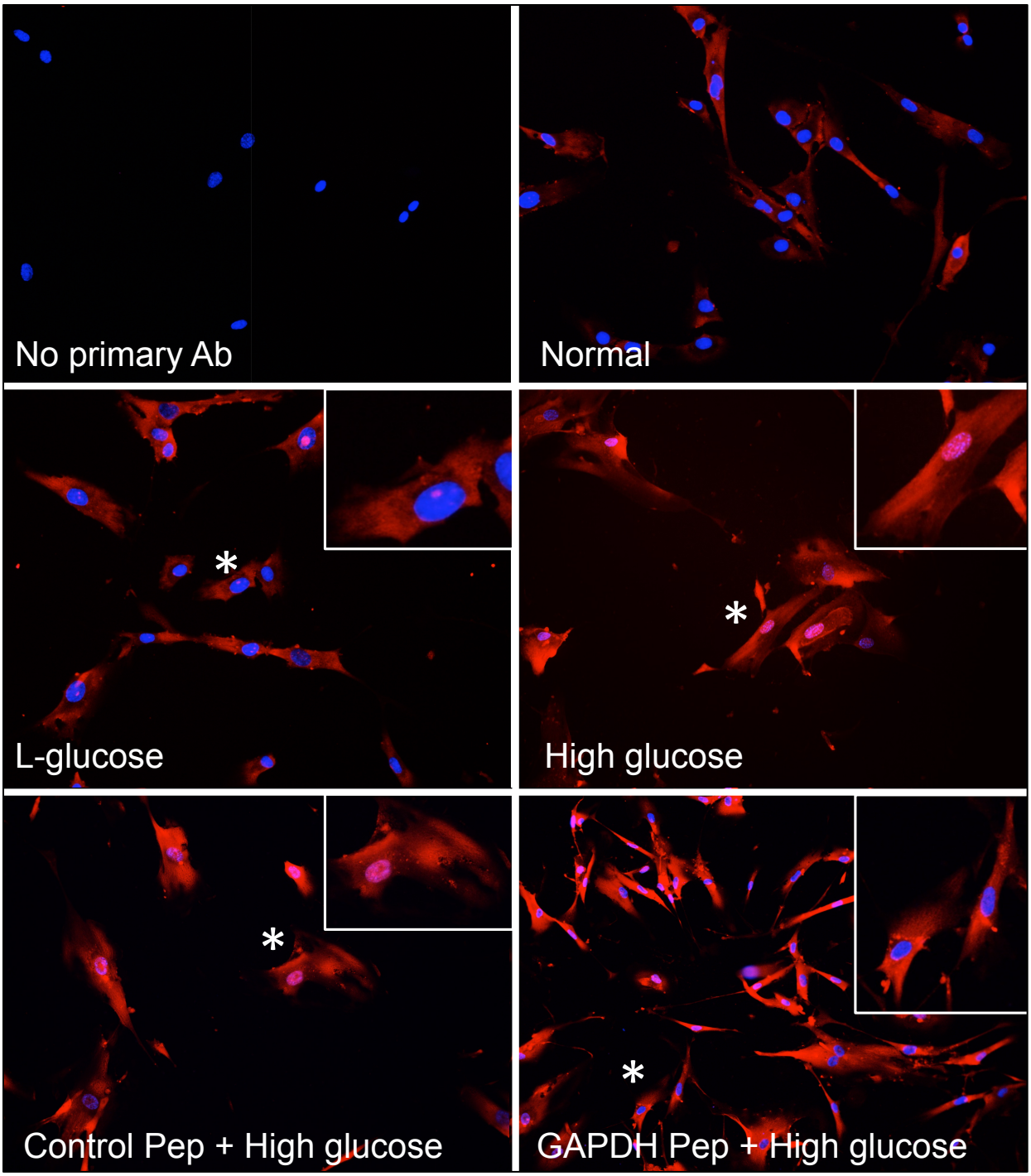


Figure 21. Immunocytochemical analysis of GAPDH nuclear translocation. HRP were treated with **top left**) no primary control (**top right**) normal glucose (5mM), (**middle left**) L-glucose (25mM) and (**middle right**) high glucose (25mM) in the presence of absence of (**bottom left**) control peptide or (**bottom right**) GAPDH peptide. GAPDH is shown in red, while DAPI- stained cell nuclei are shown in blue.

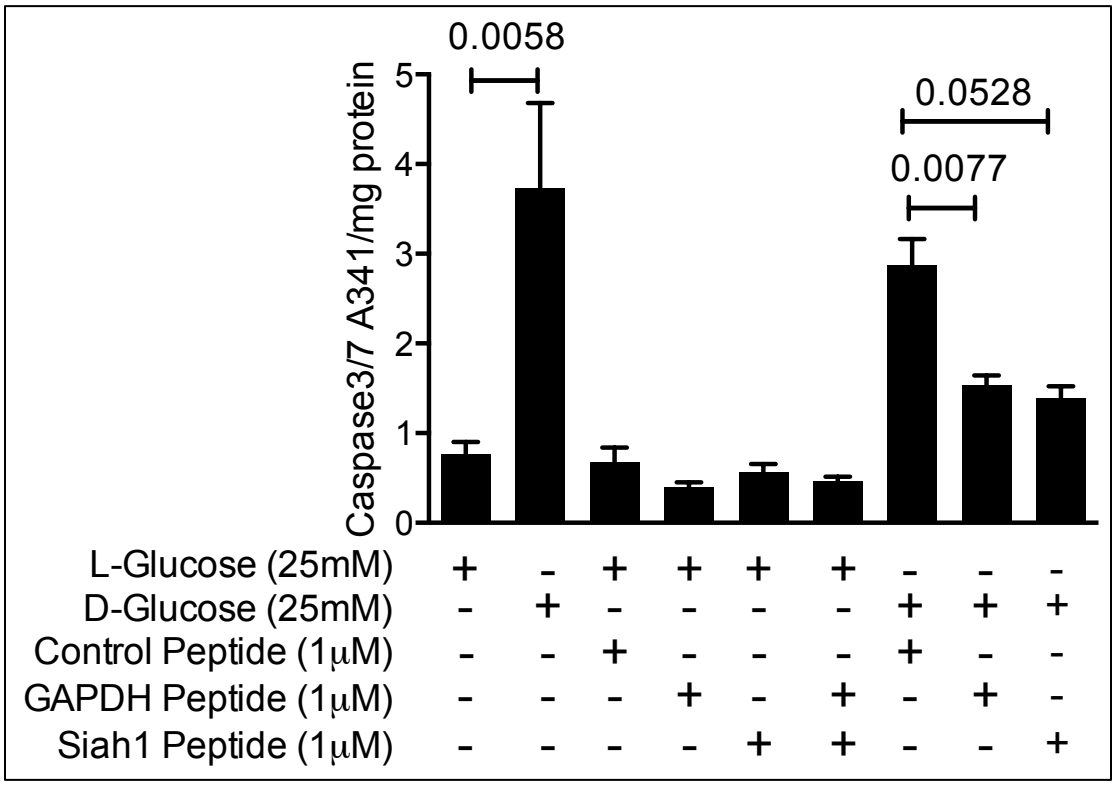


Figure 22. GAPDH/Siah1 blocking peptide inhibits high glucose-induced caspase-3 enzymatic activity. Cells were treated with L-glucose (25mM), D-glucose (25mM), Control peptide (1μM), GAPDH or Siah1 peptide (1μM). High glucose resulted in an increase in caspsae-3 enzymatic activity (P=0.0058) and this increase was significantly reduced in the presence of either GAPDH or Siah1 blocking peptide (GAPDH p=0.0077, Siah1 p=0.0528).

CHAPTER VI

Tumor necrosis factor-alpha induction of the Siah1/GAPDH pro-apoptosis pathway

After decades of research, diabetic retinopathy (DR) is now believed to result from a combination of genetic, immunological and environmental factors. Although it is widely understood that hyperglycemia is an underlying factor driving the onset of DR, other factors such as inflammation are known to play a key role in the development and progression of diabetes, and these may not be mutually exclusive [153]. Tumor necrosis factor-alpha (TNFα) is one of the main pro-inflammatory factors involved in DR [154]. This pro-inflammatory cytokine is typically expressed on the cell surface as a transmembrane protein and is cleaved into the active soluble form [155]. This cytokine has been shown to increase permeability of the endothelial cell tight junctions in the retina affecting the integrity of the blood-retinal barrier [156, 157]. Also, TNFα serum and vitreous levels have been shown to be significantly elevated in patients with diabetic retinopathy when compared to non-diabetic controls [146, 158]. Not only are TNFα levels significantly elevated during diabetes, but also Limb et al. demonstrated that all known receptors for TNFα are also significantly increased during DR. These findings suggests that TNFα activity is also significantly increased during diabetes [159]. Most importantly, several investigators have shown that TNFα levels increase as the disease progresses and worsens. For example, out of 53 diabetic patients in one study 43 had significantly elevated levels of TNFα (levels range from 4-26.4pg/ml). The highest values were acquired from patients with the advanced stage of DR known as proliferative DR or PDR [146].

Besides causing disruption of the blood-retinal barrier, TNFα is known to be a potent inducer of apoptosis [157]. Moreover, Behl et al. demonstrated in 2001 that in an STZ animal model of diabetes TNFα plays a fundamental role in microvascular cell death [103]. Since it is known that TNFα levels are significantly elevated during DR and that this pro-inflammatory cytokine plays a crucial role in blood-retinal barrier breakdown and cell death, inhibition of TNFα becomes a potential therapeutic opportunity. Currently, there are five anti-TNFα reagents clinically available to inhibit TNFα signaling. First, Etanercept is a protein composed of a TNF receptor and the Fc fragment of human IgG antibody. This drug inhibits binding of TNFα to its receptor, therefore blocking pro-inflammatory cytokine synthesis. Etanercept has been shown to inhibit pericyte and endothelial cell apoptosis *in vitro* as well as in hypergalactosemic mice [157]. Clinical trials have been indeterminate regarding Etanercept and its ability to inhibit ocular inflammation, but the use of Etanercept for treatment of diabetic retinopathy has not been examined [160].

Another inhibitor of TNFα is Adalimumab, this is a fully humanized IgG1 monoclonal antibody that binds both soluble and cell bound forms of TNFα [161]. Adalimumab has been shown to effectively treat inflammatory diseases such as juvenile inflammatory arthritis and diabetic macular edema [162, 163]. Other TNFα inhibitors include monoclonal antibodies against TNFα such as Infliximab and SIMPONI) [164-166]. To date, none of these inhibitors has been used to treat patients with DR, suggesting that further work needs to be done to elucidate the mechanism of action by which TNFα causes pericyte and endothelial cell death.

A. TNFα causes Siah1 total protein upregulation and R-Deprenyl inhibits this upregulation

In order to examine if TNFα is an inducer of the GAPDH/Siah1 pro-apoptotic pathway, hRPs were cultured in 10ng/ml TNFα. After 24hrs of treatment with experimental or control medium, cells were collected, lysed and analyzed by western blot analysis. Siah1 total protein concentrations were significantly elevated in cells cultured in TNFα when compared to control conditions. In order to determine if this upregulation is associated with the GAPDH/Siah1 pathway, R-deprenyl was administered in the absence or presence of TNFα (10ng/ml). HRPs treated with R-deprenyl in the presence of TNFα showed a slight decrease in Siah1 total protein upregulation when compared to cells treated with TNFα for 24hrs (**Figure 23**).

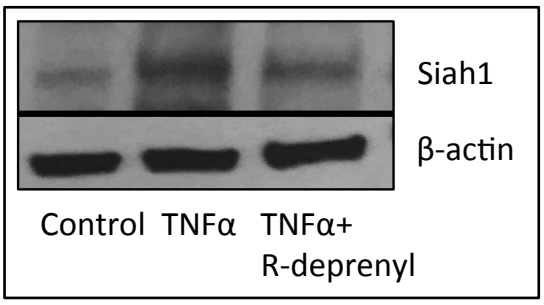


Figure 23. TNFα induces Siah1 total protein in GAPDH/Siah1 specific manner. HRPs were treated with 10ng/ml TNFα and or R-deprenyl for 24hrs. Samples were prepared for Siah1 western blot analysis. β-actin was used as a loading control.

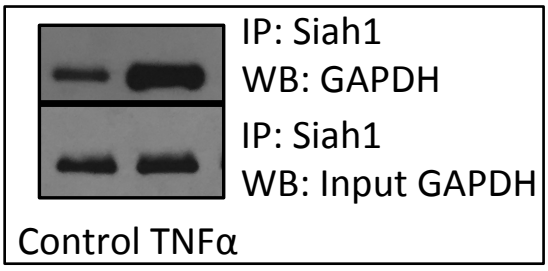


Figure 24. TNFα induces GAPDH/Siah1 association. HRPs were treated with 10ng/ml TNFα for 24hrs. Samples were prepared for Siah1 immunoprecipitation western blot analysis. Blots were probed with anti-GAPDH and anti-β-actin was used as a loading control.

B. TNFα-induced GAPDH/Siah1 association

HRPs treated with 10ng/ml TNFα also show a significant upregulation of GAPDH/Siah1 association as measured by immunoprecipitation western blot analysis (**Figure 24**). Accordingly, treatment with 10μM Siah1 siRNA or administration of 1μM GAPDH and/or Siah1 blocking peptide inhibited this TNFα-induced GAPDH/Siah1 association **(Figure 25)**.

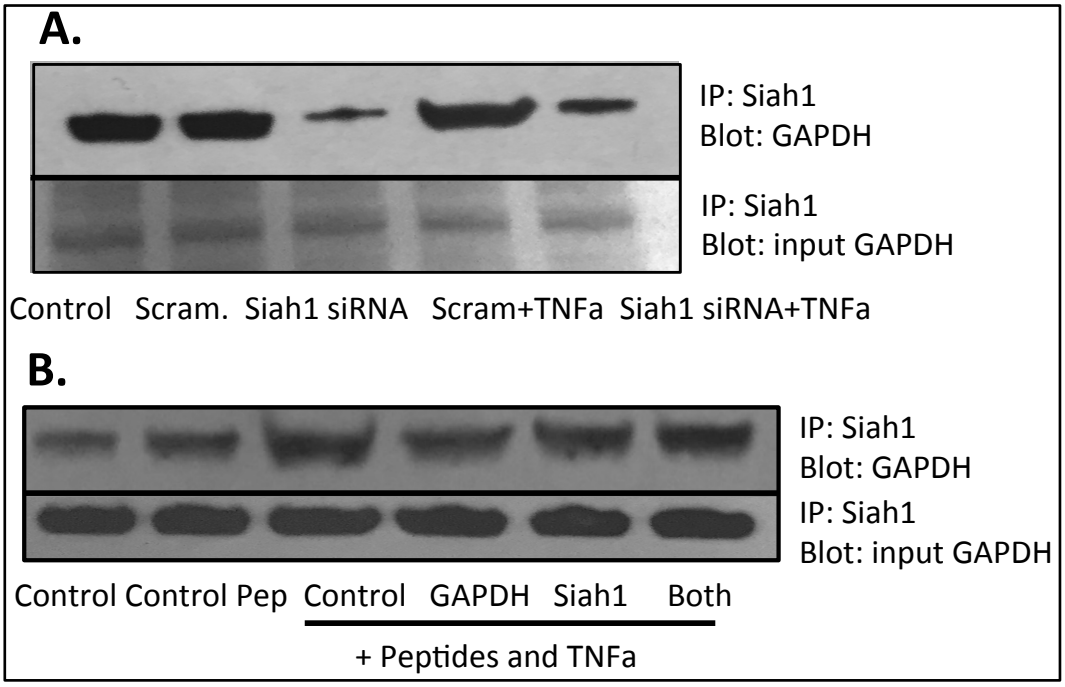


Figure 25. Siah1 siRNA and GAPDH/Siah1 peptides block TNFα-induced GAPDH/Siah1 association. HRPs were treated with 10ng/ml TNFα for 24hrs. Samples were prepared for Siah1 immunoprecipitation western blot analysis. Blots were probed with anti-GAPDH and anti-β-actin was used as a loading control.

C. TNFα-induced hRP apoptosis

Lastly, hRPs were maintained in control or TNFα (10ng/ml) culture medium for 24hrs. Caspase-3 enzymatic activity was assayed in order to examine the effect TNFα had on hRP survival. After 24hrs, TNFα treatment resulted in a significant increase in Annexin V ($p<0.0001$) and caspase-3 enzymatic activity ($p<0.0092$), suggesting that TNFα caused hRP apoptosis (**Figure 26**).

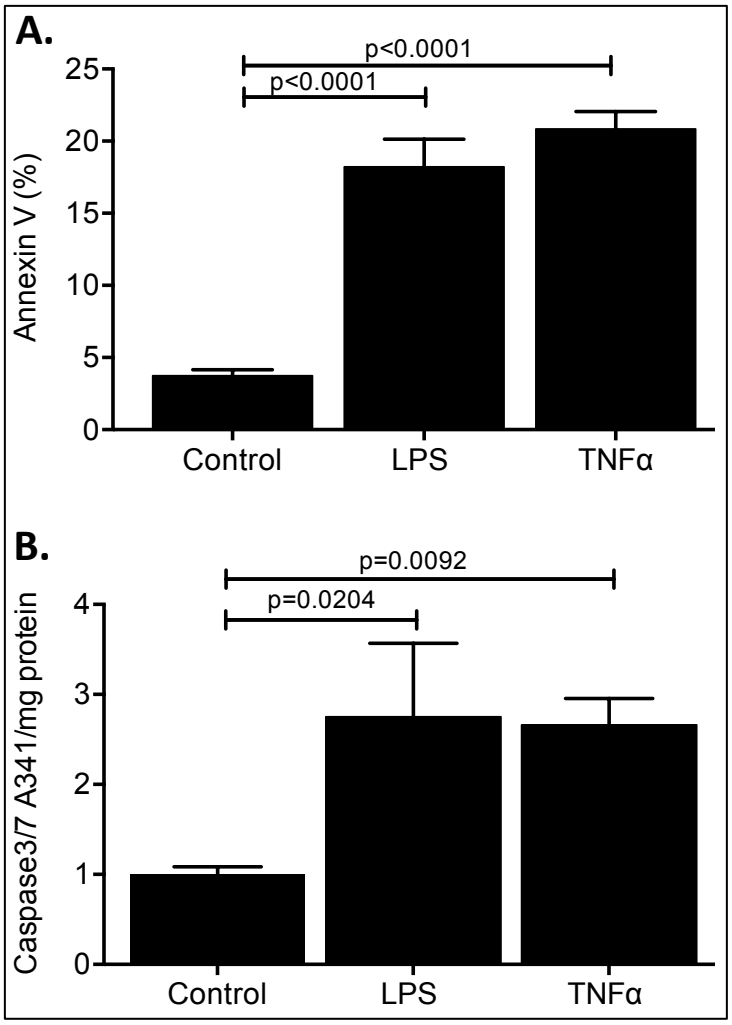


Figure 26. LPS and TNFα cause hRP apoptosis. HRPs were cultured in 10μg/ml LPS, 10 ng/ml TNFα. After 24hrs cells were collected and apoptosis was measured by Annexin V staining (**A**) or by measuring caspase-3 enzymatic activity (**B**).

While TNFα induced upregulation of Siah1 total protein and caused an increase in the association between GAPDH and Siah1, TNFα treatment did not cause GAPDH nuclear translocation in hRPs. These results suggest that other biochemical event(s) occur in the nucleus aside from GAPDH nuclear translocation, that compromise hRP survival. Classically, TNFα induces apoptosis by binding to the TNFα receptor 1, which causes assembly of the Complex II (DISC) pathway. The DISC pathway in turn results in activation of the caspase apoptotic cascade [167]. Therefore, high glucose induction of caspase-3 in hRPs treated with TNFα could be the direct result of activation of the DISC pathway instead of relying on the nuclear translocation of GAPDH.

CHAPTER VII

Imaging retinal pericyte-specific GAPDH staining

A. Animal models of DR

After discovering that the GAPDH/Siah1 pathway was involved in human retinal apoptosis the goal of the project progressed to validating these *in vitro* findings in an animal model of diabetic retinopathy (DR). The most common and well-characterized mouse model of diabetes is known as the Streptozotocin (STZ) animal model. STZ is a toxin that selectively targets and destroys insulin producing pancreatic β-cells [168]. Structurally, STZ is similar to glucose; therefore, it gets transported into the cell by the same transport protein, known as GLUT2. β-cells inherently have higher amounts of GLUT2 receptor when compared to other cells in the body making them particularly susceptible to the STZ toxin [169]. Although STZ is technically a model of Type 1 diabetes, this model is routinely used as a DR model since it has been shown to consistently recreate human DR associated pathology [170]. For instance, vascular changes have been reported to occur at 8 days of after hyperglycemia induction [171]. These changes lead to vessel leakage, thickening of capillary basal lamina, and after six to nine months of hyperglycemia, pericyte ghosts and avascular capillaries are evident [172-175]. Alloxan is also another toxin used to selectively poison pancreatic β-cells, resulting in diabetic animals [176, 177].

Furthermore, several genetic animal models are also commonly used to model diabetes *in vivo*. The db/db leptin deficient mouse model inhibits leptin receptor activity modeling type 2 diabetes [178]. Leptin is a hormone responsible for regulating appetite and controlling food intake [179]. Vascular abnormalities like pericyte loss, acellular

capillaries and blood-retinal barrier breakdown can be seen around 2 months in these diabetic obese mice [180-182]. Another model that manipulates the leptin receptor is the ob/ob leptin resistant mouse model [183]. In this model the leptin receptor is mutated causing a decrease in leptin production. Consequently, these mice also become obese, diabetic and exhibit similar abnormalities to those seen in diabetic patients.

The non-obese diabetic (NOD) mouse model is also used to model the diabetic complications seen in human patients. In this model pancreatic β-cells are selectively destroyed by infiltrating T-cells [184]. Consequently, there is a selective loss of β-cells from the pancreas modeling events that occur during diabetes. This mouse model can be used to model both Type 1 and type 2 diabetes [177].

B. GAPDH nuclear translocation *in vivo*

The presence and/or activation of the pericyte specific GAPDH/Siah1 pathway has not been previously investigated *in vivo* thus, it is of great interest to examine whether this pathway is also playing a role in animal models of DR. Validation of targets using animal models are crucial when developing potential targets to test in clinical trials. Since diabetic retinopathy occurs after years of diabetes, animal models also need to mimic this long period of hyperglycemia. Accordingly, most animal models of DR are inherently time consuming since you need to wait 6-8 months to see retinal pericyte death [185]. Although STZ animals are available for purchase these mice are very costly. Since the GAPDH/Siah1 pathway has not previously been studied in the context of pericyte apoptosis *in vivo*, an acute model of DR was used. Being able to use

an acute model rather than making animals diabetic for six-nine months will yield preliminary data needed to justify the longer time points.

In order to acutely induce DR *in vivo*, mice were injected with 10ng/ml LPS and 50ng/ml TNFα for 24hrs. LPS is an endotoxin, which is known to induce a potent inflammatory response. As previously stated inflammation has been shown to play a key role during early stages of DR. Therefore by using stimuli that elicit inflammatory responses *in vivo* can be used to model events that occur in diabetic patients. Also, both LPS and TNFα cause hRP apoptosis, making them valuable tools to acutely assay whether the GAPDH/Siah1 pathway is playing a role *in vivo* (**Figure 27**). After intravitreally injecting mice with either TNFα or LPS, after 24hrs retinas were dissected and stained with anti-neuron glial 2 (NG2) and anti-GAPDH antibodies. NG2 was used a marker to identify pericyte specific GAPDH nuclear translocation. GAPDH translocation is a crucial step in the activation of apoptotic cell death in the GAPDH/Siah1 pathway. Thus, in order to establish therapies designed to inhibit high glucose-induced apoptosis, GAPDH nuclear translocation and accumulation needs to be determined.

Preliminary data from TNFα or LPS injected animals demonstrates no difference between control animals and those injected with either inflammatory stimuli (**Figure 27**). Although there is no significant difference between control and experimental groups, the protocol established to image pericyte specific GAPDH nuclear translocation will make it possible to determine if the GAPDH/Siah1 pro-apoptotic pathway is playing a role in pericyte apoptosis *in vivo*. Future work needs to be done in order to optimize the *in vivo* parameters used in these preliminary studies. Hence, the protocols developed in this

dissertation will allow for future studies to determine a role of the GAPDH/Siah1 pathway in animal models of DR.

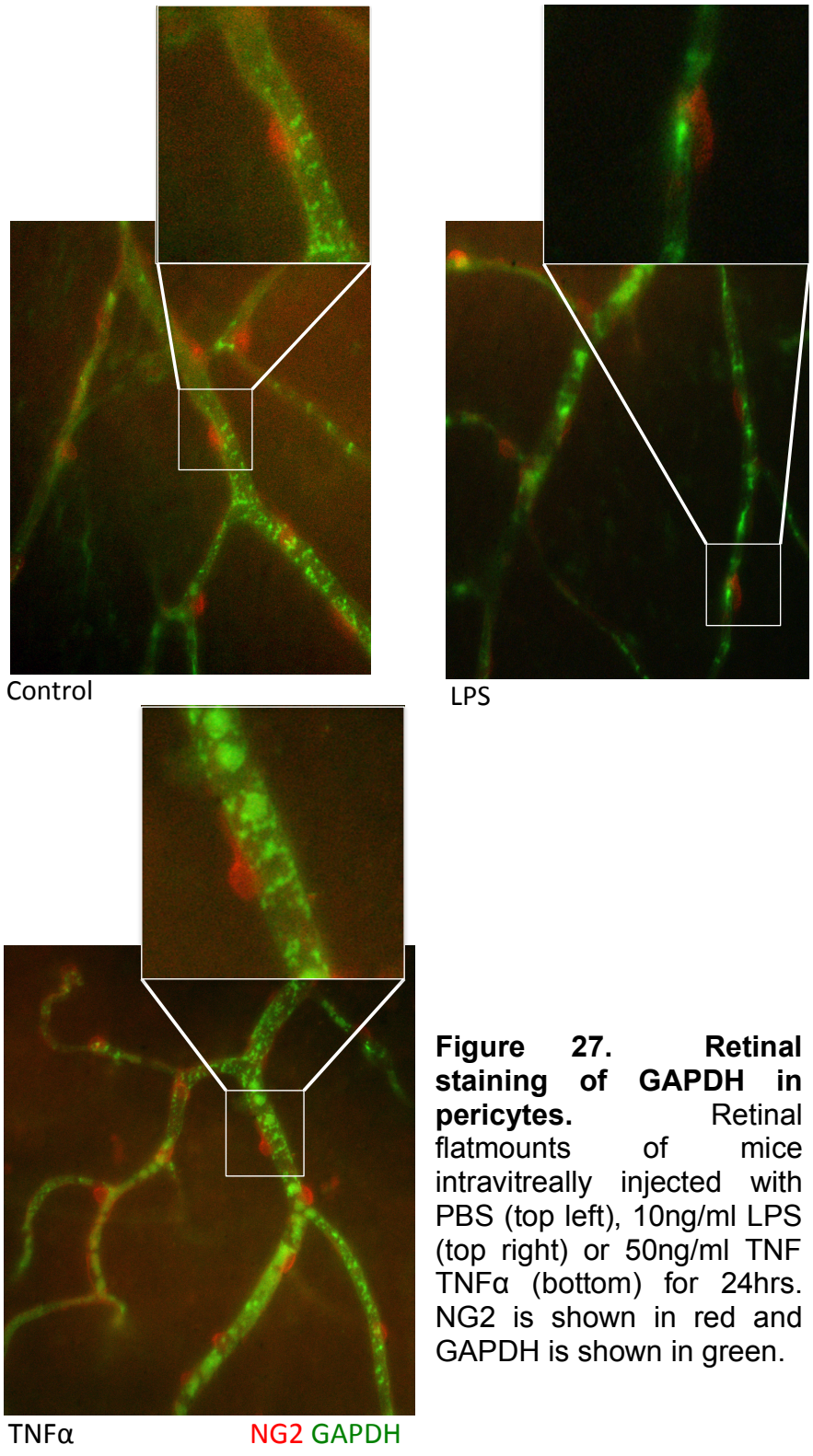


Figure 27. Retinal staining of GAPDH in pericytes. Retinal flatmounts of mice intravitreally injected with PBS (top left), 10ng/ml LPS (top right) or 50ng/ml TNF TNFα (bottom) for 24hrs. NG2 is shown in red and GAPDH is shown in green.

CHAPTER VIII

Conclusions and future directions

In summary, the work presented in this dissertation provides evidence for a role of the GAPDH/Siah1 pro-apoptotic pathway in hRP cell death under diabetes-relevant conditions. More importantly, this work aims to fill the knowledge gap between the molecular events that connect hyperglycemia and retinal cell death. Although it is widely acknowledged that the selective loss of human retinal pericytes from the vasculature occurs during early stages of diabetic retinopathy (DR), the molecular mechanisms that regulate this cell loss still remain unknown.

Several decades of research on DR has led to the discovery of various signaling pathways hypothesized to play a role in advancing DR pathology. One of these pathways involves the upregulation of AGEs as a result of hyperglycemia. Interestingly, Du et al. demonstrated that hyperglycemia-induced overproduction of mitochondrial superoxides induces a potent decrease in GAPDH enzymatic activity [186]. AGEs have also been shown to increase reactive oxygen species (ROS) production, and as a result inhibit GAPDH enzymatic activity [187]. Intriguingly, it is hypothesized that NO-induced S-nitrosylation abolishes GAPDH catalytic activity making GAPDH suitable to bind to Siah1 [120]. This binding in turn allows for the translocation of the pathway to the nucleus. Other groups have also shown that ROS levels increase as nuclear levels of GAPDH rise. Taken together these studies suggest that AGE-induced up regulation of ROS could also be activating the GAPDH/Siah1 pathway causing cell instability and death [115, 120]. Future studies are required in order to examine if there are commonalities in the GAPDH/Siah1 pathway and the ROS-induced AGE cell death

pathway. Connecting the GAPDH/Siah1 pathway to previously suggested pathways is beneficial since there are currently two AGE inhibitors that have been shown to slow and even prevent the progression of diabetic retinopathy in animal models of DR [188, 189]. Of particular interest is the AGE inhibitor, aminoguanidine, which has recently been shown to prevent the progression of diabetic retinopathy in Type 1 diabetic patients [190]. Therefore, understanding where overlap between the GAPDH/Siah1 pathway and increased levels of AGEs exists may allow for a more detailed understanding of the molecular mechanisms occurring during early stages of DR. Moreover, drugs designed to block AGEs could also be advantages in blocking diabetes-induced retinal pericyte cell death.

The importance of the GAPDH/Siah1 pathway has also been demonstrated in retinal glial cells [124]. Inhibition of this pathway with Siah1 siRNA and R-deprenyl prevents high glucose-induced retinal cell death in this other cell type. Unlike pericytes that are known to die at a very early stage of DR progression, retinal glial cells are believed to undergo apoptosis at a much later stage of DR. Thus, early inhibition of the GAPDH/Siah1 pathway could prevent later apoptotic events seen in other retinal cells like the retinal glial cells.

Not only is inhibiting the GAPDH/Siah1 pathway beneficial to preventing retinal pericyte and retinal glial apoptosis but it could also be advantageous in preventing endothelial cell death. It is well documented that retinal pericytes and endothelial cells have a close and intimate relationship. They communicate physically through tight junctions but they also communicate by integrating signals along the vessel to promote vessel stability and health [191]. Hence, it is plausible that the GAPDH/Siah1 pathway

plays a role in endothelial cell death as well. Similarly, it is important to access the specificity of the GAPDH/Siah1 pathway in order to determine potential off target effects associated with inhibiting this pathway *in* vivo. In order to determine the specificity of the GAPDH/Siah1 pathway to human retinal pericytes, human retinal microvascular endothelial cells (hRMEC) and human dermal fibroblasts (hDF) were cultured in high glucose. As shown in **Figure 28A** there was no significant difference in Siah1 total protein levels in hRMEC or hDF after treatment with high glucose. There is also no significant difference in the amount of nuclear GAPDH present in hDF after 48hrs (**Figure 28B**). It is important to note that after 48hrs high glucose treatment also does not cause GAPDH nuclear translocation in hRMECs. These results coupled with the observation that glucose exposure for 48hrs does not result in hDF or hRMEC cell death suggests that the GAPDH/Siah1 pathway in the retina could be pericyte and glial cell specific (**Figure 28C**). An intensive comprehensive study of other retinal cells needs to be completed in order to confidently state the specificity of the GAPDH/Siah1 pathway to retinal pericytes. Since the GAPDH/Siah1 pathway seems to be specific to pericytes and glial cells the potential deleterious effects on other cells types after inhibiting this pathway are slightly reduced.

Although the GAPDH/Siah1 pathway does not seem to be playing a role in directly mediating endothelial cell death, inhibition of this pathway in pericytes has the potential to indirectly affect endothelial cell apoptosis. As previously stated in **Chapter I section D** in order to maintain vascular stability a healthy relationship between retinal pericytes and endothelial cells is essential and necessary. For example, it is well documented that the same diabetes relevant stimuli (e.g. high glucose) that cause

retinal pericyte death also cause endothelial cell death [192, 193]. It is also known that pericytes control endothelial cell proliferation by modulating levels of TGFβ [194]. These results coupled with other work demonstrating the intensive cross talk that occurs between endothelial cells and pericytes strongly suggests that pericyte death could be strongly correlated with a loss of endothelial cells from the vasculature [73, 76, 78-80, 191, 195]. Therefore, elucidating mechanisms that prevent pericyte cell death might also prove beneficial in preventing downstream endothelial cell loss.

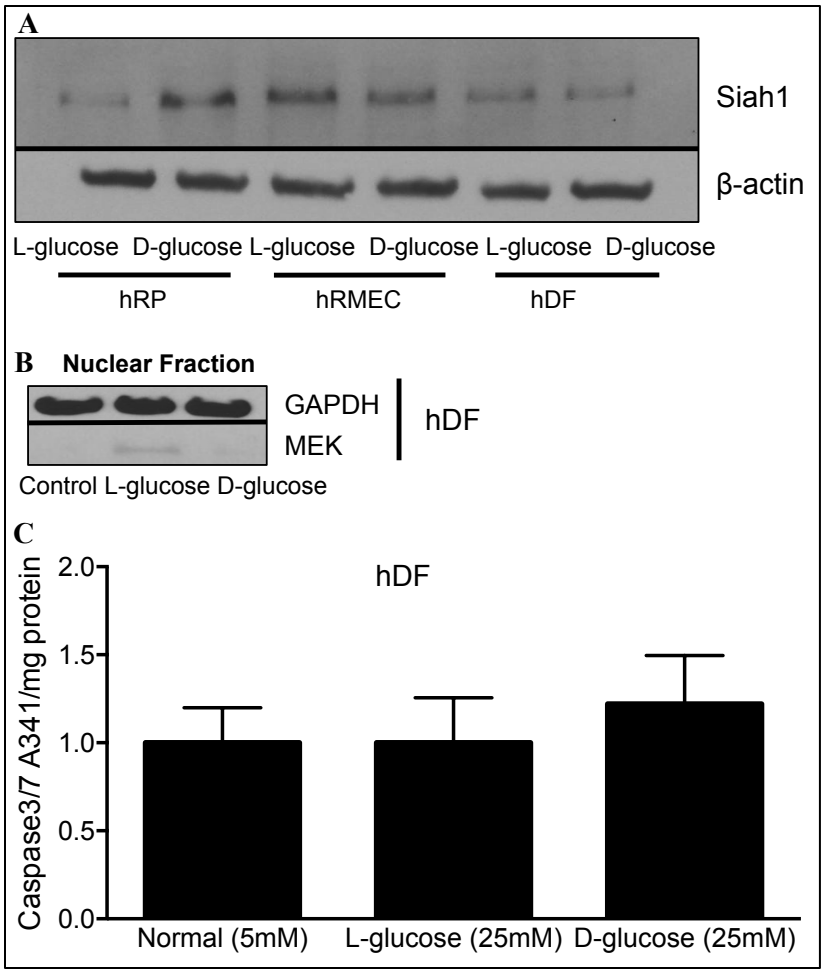


Figure 28. GAPDH/Siah1 complex in human retinal pericytes (HRP), human retinal microvascular endothelial cell (hRMEC) and human dermal fibroblast (hDF). (**A** Siah1 western blot analysis (**B**) GAPDH nuclear fractions and (**C**) Caspase-3 enzymatic activity assay of hDFs treated with high glucose for 48hrs. High glucose (48hrs) does not cause GAPDH nuclear translocation or cell death in hDFs or hRMECs.

To our knowledge this study marks the first attempt to elucidate a role for the pro-apoptotic GAPDH/Siah1 in human retinal pericyte cell death. Based on previously published data demonstrating high glucose-induced nuclear accumulation of GAPDH and the findings of the present experiments, the work presented in this dissertation proposes a model that is initiated by an increase in pericyte NO levels as the result of high glucose. This increase in NO levels leads to an increase in S-nitrosylated GAPDH facilitating the association between GAPDH and Siah1. It is important to note that increased levels of the GAPDH/Siah1 complex could also result from an increase in Siah1 availability. Normally, Siah1 total protein levels are relatively low when compared to pathological levels. The work presented in this dissertation along with published results from work done in retinal glial cells demonstrates that high glucose is one inducer of Siah1 upregulation [124]. Thus, high glucose could be increasing the association between GAPDH and Siah1 by increasing Siah1 new protein synthesis along with causing an increased in post translationally modified GAPDH. Further studies need to be conducted in order to fully depict the molecular events responsible for specifically causing an increase in GAPDH/Siah1 binding and association.

Although the *in vitro* work presented here suggests that the GAPDH/Siah1 pathway is playing a prominent role in inducing retinal cell death, there are several aspects of this project that need further development. For instance, the ability to inhibit pericyte apoptosis in patients recently diagnosed with DR has the potential to prevent the progression of this vision threatening disease. Currently, R-deprenyl is primarily used to treat early stages of Parkinson's disease [151]. This drug is commonly used in combination with other drugs used to retard progression of Parkinson's disease. Several

reports suggest that R-deprenyl slows the progression of Parkinson's disease by inhibiting the death of dopamine-generating cells. During this debilitating disease, death of dopamine producing cells leads to the degeneration of the central nervous system [196]. In turn patients with Parkinson's suffer from severe loss of motor and cognitive function. The specific molecular mechanism responsible for the death of dopamine generating cells still remains unknown and is active area of research in this field.

Based on the anti-apoptotic role of R-deprenyl this drug could also prove useful in preventing pericyte loss during early stages of DR. Since R-deprenyl is routinely used in the clinic, it has successfully passed rigorous safety and efficacy studies. Therefore, adopting R-deprenyl for the treatment of DR could prove advantageous since it is known that R-deprenyl is safe and well tolerated when administered to patients. Due to the fact that there is only evidence that R-deprenyl prevents high glucose-induced pericyte apoptosis *in vitro* a significant amount of work must be done in order to translate these events to an animal model of DR. Based on *in vitro* work suggesting that R-deprenyl inhibits cell death in cultures, this suggests that R-deprenyl has the potential to prevent the loss of early pericytes from the vasculature. Thus, R-deprenyl has the ability to become a plausible therapeutic solution for stopping progression of DR. It would also be interesting to do an analysis on diabetic Parkinsons patients receiving R-deprenyl and analyze the effect of deprenyl to slow the progression of diabetic retinopathy. These patients would also contribute interesting information on the effectiveness of R-deprenyl as a treatment for diabetic retinopathy.

Besides validating the efficacy of using R-deprenyl to inhibit pericyte cell death in animal models of DR, validation of Siah1 siRNA and blocking peptides is also needed.

Out of the three inhibitory methods used to inhibit the GAPDH/Siah1, use of the GAPDH or Siah1 blocking peptide, or a combination, *in vivo* are the most encouraging. Since these TAT-FLAG peptides are specifically designed to target GAPDH/Siah1 binding the potential for off target effects is somewhat reduced. Design of therapies with a known mechanism of action coupled with a low risk of harmful side effects is ideal. Future *in vivo* studies are necessary in order to evaluate the therapeutic potential of targeting the GAPDH/Siah1 pathway in early stages of DR. Future studies are also needed in order to further understand adverse side effects associated with inhibiting retinal GAPDH/Siah1 signaling.

In addition to further characterization and validation of the tools used to inhibit the GAPDH/Siah1 pathway, future work needs to be done in order to better understand the nuclear events that occur after the GAPDH/Siah1 complex successfully translocates from the cytosol to the nucleus. Preliminary results demonstrate a significant increase in p53 relative expression in HRP cultures exposed to D-glucose when compared to either control or osmotic control conditions. This upregulation in p53 as a result of high glucose is consistent with previous work done by the Mohr group looking at the GAPDH/Siah1 pathway in retinal glial cells. Interestingly, inhibition of the GAPDH/Siah1 association blocks high glucose-induced p53 upregulation (**Figure 29**). Siah1 has also been shown to act as a p53 inducible gene in other model systems [197, 198]. Recently, it has been shown that after nuclear translocation of the GAPDH/Siah1 complex, GAPDH formed a complex with p53 and activated p53-dependent cell death [199]. Since tumor protein p53 is normally involved in cell cycle arrest and apoptosis, upregulation of p53 could explain a mechanism by which the GAPDH/Siah1 pathway

plays a role in high glucose-induced HRP apoptosis. More targeted inhibition of the GAPDH/Siah1 pathway (Siah1 siRNA or GAPDH/Siah1 blocking peptides) must be conducted in order to verify the involvement of the GAPDH/Siah1 in p53-induced cell death. **Figure 30** demonstrates a proposed mechanism by which high glucose may cause pericyte death in a GAPDH/Siah1-specific manner.

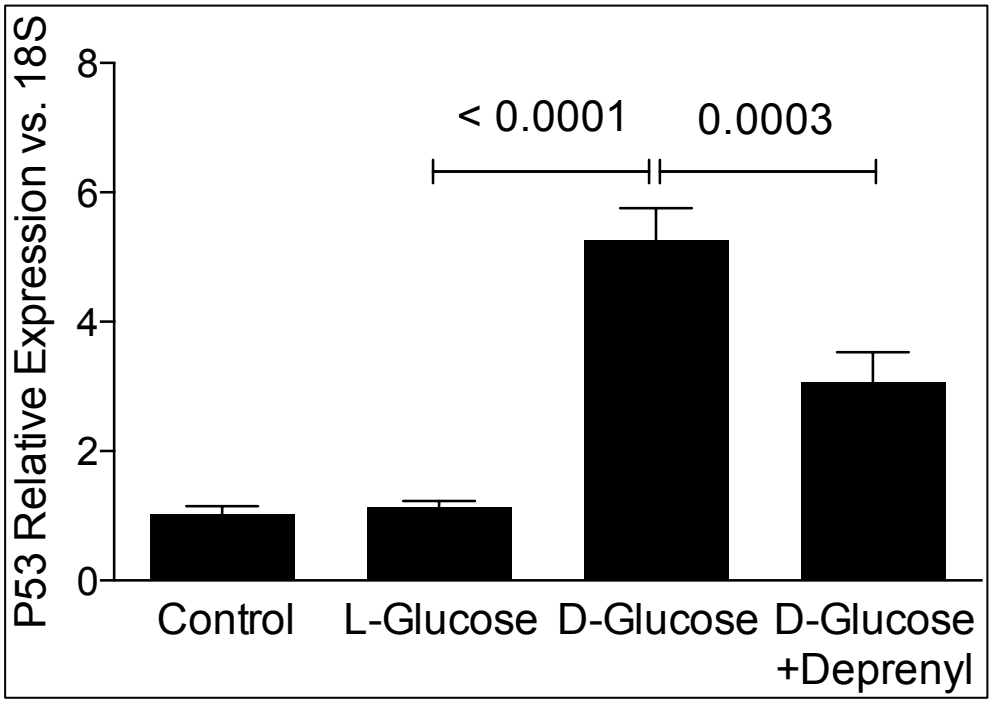


Figure 29. Deprenyl inhibits high glucose-induced p53 upregulation. Human retinal pericytes were cultured in either normal (Control) glucose, L-glucose (25mM) or D-glucose (25mM). Some cultures also received treatment of 1nM R-deprenyl in the presence of D-glucose. Cells were collected and analyzed via RT-PCR analysis. Graph demonstrates p53 relative expression vs. 18S. 18s serves as a loading control. High glucose causes a significant increase in p53 expression ($p<0.0001$) when compared to control conditions. R-deprenyl blocks this high glucose-induced p53 upregulation ($p=0.0003$).

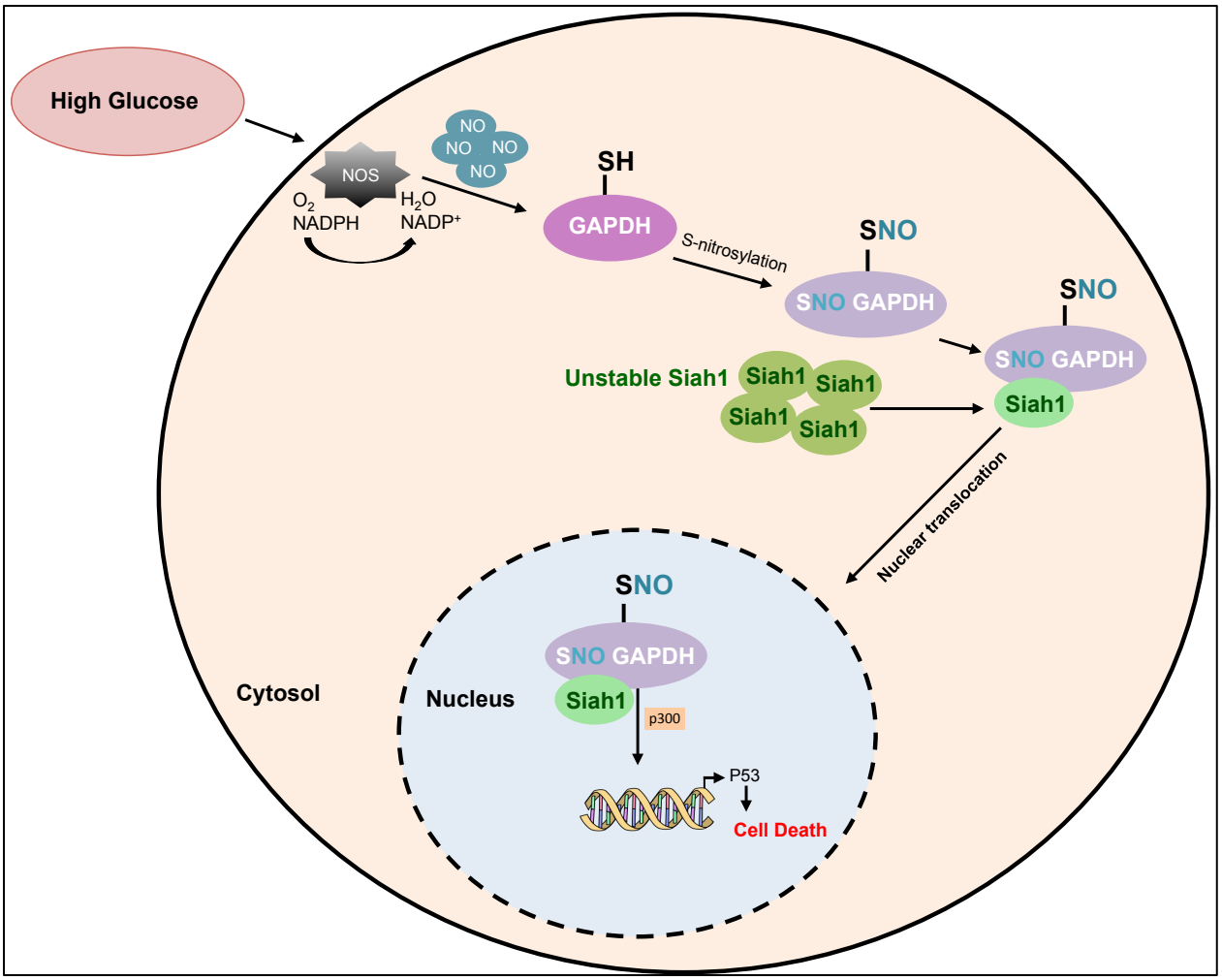


Figure 30. GAPDH/Siah1 pathway in human retinal pericyte apoptosis. Proposed model of the pro-apoptotic pathway GAPDH/Siah1 in high glucose-induced human retinal pericyte apoptosis. Cell stress, such as high glucose, causes an increase in nitric oxide synthesis (NOS) activity. This increase in NOS activity results in elevated cytosolic nitric oxide (NO), which causes S-nitrosylation of GAPDH. Nitrosylated GAPDH associates with Siah1, stabilizing the complex and facilitating its translocation to the nucleus. Once in the nucleus, Siah1 degrades target proteins and/or GAPDH undertakes other non-glycolytic functions resulting in cell instability and ultimately cell death. This model is an adaptation from Hara et al. 2005. Ref [120]

Furthermore, in order to further understand the nuclear events occurring in HRPs exposed to chronic levels of D-glucose, identification of GAPDH's transcriptional targets is necessary. As previously mentioned in **Chapter II,** GAPDH is known to have transcriptional factor capabilities under certain circumstances. GAPDH's transcriptional activity was demonstrated in 2003 when Zheng et al. showed activation of the histone H2B promoter by a complex including GAPDH. In fact, GAPDH is an essential component of the OCA-S coactivator complex responsible for histone activation [200]. Nuclear GAPDH has also been shown to play a role in RNA export by binding to transfer RNA in a sequence specific manner [201]. Lastly, there is evidence that GAPDH binds to the p300/CBP pathway causing activation of p53 and p53-related genes such as PUMA [129]. Since there is ample evidence suggesting GAPDH's DNA binding capabilities in other systems, it would be beneficial to examine GAPDH's nuclear targets in HRPs exposed to high glucose for an extended period of time. Tools such as chromatin immunoprecipitation sequencing can be used to determine GAPDH's specific nuclear targets. Identification of these targets can result in more opportunities to target and block high glucose-induced pericyte apoptosis.

The last ongoing aspect of this project is to translate *in vitro* results presented here to animal models of DR. Determining the relevant stimuli to model a chronic condition that occurs in humans is always difficult. This is especially true when designing animal experiments to model diabetic retinopathy. The first step in validating the *in vitro* findings demonstrated in **Chapters IV-VI** is to establish the presence and activation of the GAPDH/Siah1 pathway *in vivo*. Future work must be done in order to optimize the effect of diabetes-relevant stimuli on GAPDH nuclear translocation *in vivo*.

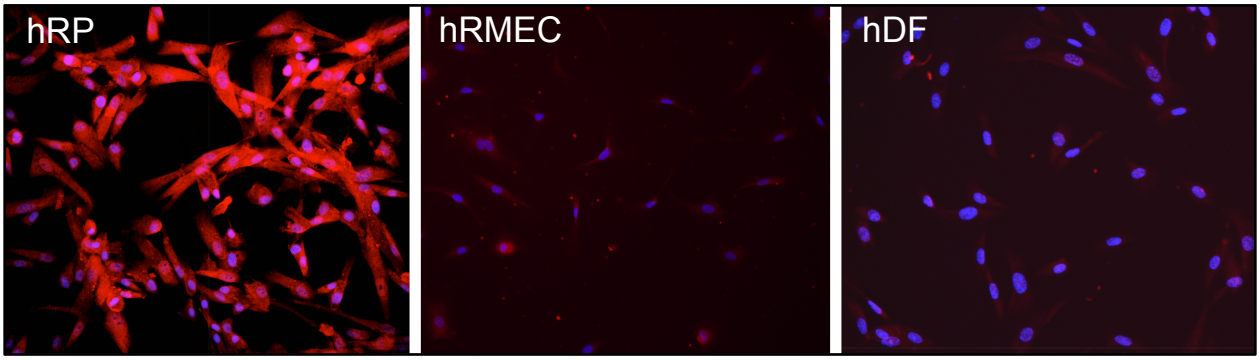
For instance, LPS may cause GAPDH nuclear translocation at different times and doses than the ones shown in **Chapter VII**. Also, since the GAPDH/Siah1 pathway is initiated by increased NO levels, agents that specifically induce NO accumulation could be potent inducers of this pathway *in vivo.* Other groups have shown induction of the GAPDH/Siah1 pathway by treating cells with NO donors such as S-nitroso-glutathione (GSNO) [202]. Also, the Mohr group nicely demonstrated a significant increase in GAPDH nuclear translocation in Müller cells after treatment with interleukin-1 beta (IL-1β) [203]. Interestingly inhibition of IL-1β production was sufficient to significantly reduce GAPDH nuclear accumulation. IL-1 β has been shown to play a significant role in beta cell mass loss and activation of inflammatory cytokines in type 2 diabetes [204]. Also, IL-1β has been shown to induce nitric oxide synthase activity and nitric oxide formation [205]. Thus, injecting mice with IL-1β could also be a method to acutely induce the GAPDH/Siah1 pathway *in vivo*. Subsequent studies should be designed to determine the effectiveness of inhibiting the GAPDH/Siah1 pathway as a preventative measure to halt DR progression. Future studies are also necessary to determine how often these peptides need to be administered to meaningfully prevent hyperglycemia-induced cell death. These studies will make it possible to determine how often patients will need to treated with these TAT-FLAG blocking peptides. After determining the accurate time course and dosage of each treatment (either Siah1 siRNA or TAT-FLAG GAPDH/Siah1 peptides) retinal pericyte loss can be determined using retinal digest analysis (**Appendix E**). This technique is commonly used to determine pericyte loss *in vivo* [206] and will provide the necessary *in vivo* evidence needed to translate these results to the clinic.

Furthermore, it is important to highlight concerns associated with treating a chronic disease like diabetes. The Center for Managing Chronic Disease defines a chronic disease as a long lasting condition that can be controlled but not cured and affects the population worldwide. Designing treatments for chronic diseases such as diabetes is particularly challenging because long-term administration of drugs can result in unwanted complications such as liver and kidney disease [207]. Treating DR over long periods of time is also particularly challenging because most of the available therapies are administered via intravitreal injection. For example, one of the side effects associated with intravitreal injection of anti-VEGF is endopthalmitis. This condition is usually caused by infection and is characterized as an inflammation that occurs in intraocular cavities. Ideally, TAT-FLAG GAPDH/Siah1 blocking peptides would be administered directly to the eye to eliminate systemic off target effects associated with inhibition of GAPDH and Siah1, therefore risk of inflammation and other side affects associated with intravitreal injection are valid concerns when proposing these peptides as treatments for DR.

In essence, this project has identified a novel mechanism connecting diabetic conditions and pericyte death via GAPDH nuclear translocation. The knowledge gained from this work will guide future efforts to develop novel and effective therapies for early assessment and treatment of DR.

APPENDIX A

Immunocytochemistry of NG2 staining is shown in red and nuclei are stained with DAPI in blue. Human retinal pericytes (hRP), human retinal microvascular endothelial cells (hRMEC), human dermal fibroblast (hDF).



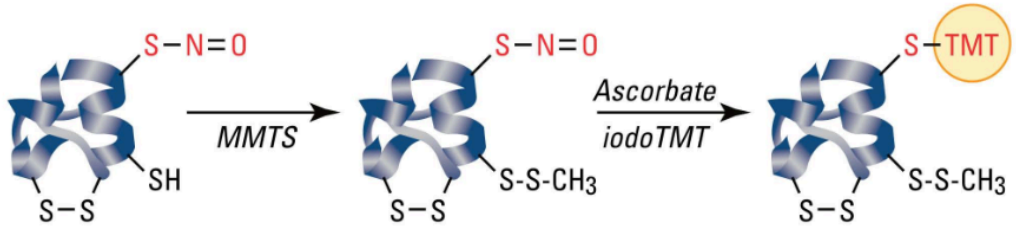
APPENDIX B

siRNA oligomer sequences.

Name	Strand	Sequence
Negative Control Catalog: 1022076	Sense	5'-UUCUCCGAACGUGUCACGUTT-3'
	Antisense	5'-ACGUGACACGUUCGGAGAATT-3'
Siah1 siRNA A Sc-37495A	Sense	5'-CAGCAUAAGUCCAUUACAATT-3'
	Antisense	5'-UUGUAAUGGACUUAUGCUGtt-3'
Siah1 siRNA B Sc-37495B	Sense	5'-GCAACAGCCAUUAUGAAUAtt-3'
	Antisense	5'-UAUUCAUAAUGGCUGUUGCtt-3'
Siah1 siRNA C Sc-37495C	Sense	5'-GUGUUGAAAUGGCAAUCAAtt-3'
	Antisense	5'-UUGAUUGCCAUUUCAACACtt-3'

APPENDIX C

Diagram of Thermo scientific pierce s-nitrosylation western blot kit.



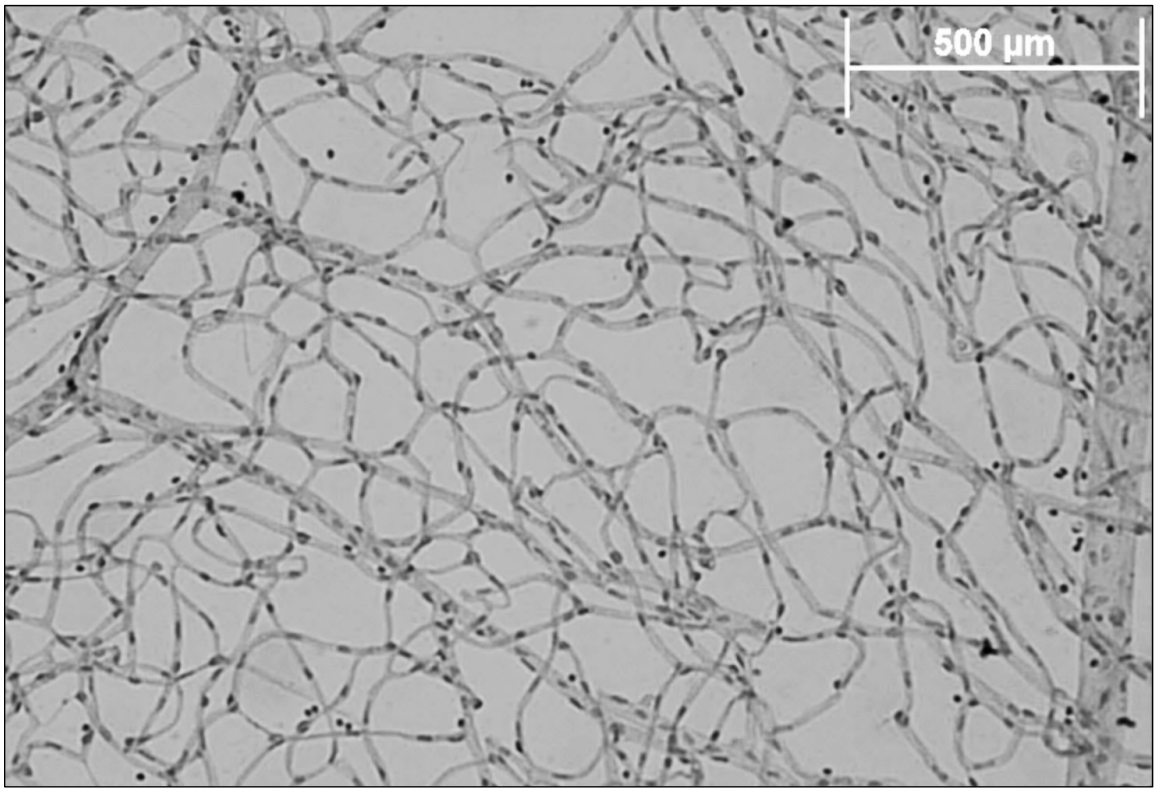
APPENDIX D

Taqman gene expression IDs used in qRT-PCR.

Gene Name	ID	Species
ACTB	Hs99999903_m1	Human
Siah1	Hs02339360_m1	Mouse

APPENDIX E

Represent image of a C57Bl/6 mouse retinal digest in control conditions.



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