EFFECTS OF GLUCOSE AND FLOW ON REACTIVE OXYGEN SPECIES IN BRAIN ARTERY ENDOTHELIAL CELLS

by

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Abstract

Endothelial cells play a vital role in the normal physiology of the vasculature. The cerebrovascular region is highly populated by endothelial cells with distinct morphology and functions. However, endothelial cells are also a vital region in the pathophysiology of the vasculature, such as aneurysm formation, due to reactive oxygen species (ROS) production. To study the effects of glucose and flow on ROS production in brain arterial endothelial cells, ROS production was measured. This thesis is divided into three parts: glucose effect on ROS, flow effect on ROS, and glucose effect on flow-induced ROS. Previous endothelial cultures were provided by Joeseph Moran-Guiati and Jason Kushner. The effect of high glucose on static endothelial cells was shown to increase ROS production as compared to the effect of normal glucose. Under chronic treatment of endothelial cells with high flow, ROS production was significantly greater that in endothelial cells under chronic treatment of normal flow. High glucose was shown to exacerbate the high flow response. These studies provide insight to a possible connection between intracranial aneurysm formation and a major risk factor, Diabetes Mellitus.

Introduction

Diabetes Mellitus (DM) serves as a major risk factor for many micro- and macrovascular pathologies such as intracranial aneurysm formation (IA), nephropathy, and retinopathy[1]. Patients with DM experience elevated blood glucose levels on a constant basis[1,2]. Glucose can affect cellular processes such as reactive oxygen species (ROS) production[1,2], methylglyoxal formation[3-7], and PKC activation[8,9].

The endothelium is thought to be the major early modulator to the pathogenesis of macrovascular diseases such as IA formation/damage. In 2011, Nicholas Liaw found that early IA development and damage was due to ROS production within brain artery endothelial cells via tempol administration to IA-induced rabbits. Upon investigating the result of damage to the blood vessel, it was found that the endothelium was intact during the early stages of pathogenesis of IA formation while the tunica media thinned causing a weakness in the blood vessel. Therefore, they hypothesized that ROS produced within the endothelial cell exits and causes damage to the basement membrane, internal elastic lamina, and the tunica media[10].

The pathogenesis of IA is also in response to the changes in wall shear stress (WSS) on the endothelium[11]. Baseline flow occurs in straight blood vessels where laminar flow produces between 1.5 and 2.5 Pascals (Pa) of shear stress[11]. High WSS typically occurs on the flanking walls of arteries at regions of bifurcations and produces around 10 Pa of stress[11]. WSS has been shown to stimulate ROS production through PECAM-1, an adhesion molecule on the membrane of endothelial cells[12-17].

This research aims to characterize the effects of glucose and flow on cultured bovine brain arterial endothelial cells (BBEC). I believe that both high glucose and high flow will

significantly increase intracellular ROS production compared to normal glucose and normal flow.

Background

Organization and Function of Brian Arteries

Brain arteries run throughout the entire cranium bringing oxygen and nutrient-rich blood to cranial structures. Arteries pertaining to the anterior and posterior circulation of blood within the cerebrum and surrounding structures are associated with a region called the circle of Willis. The circle of Willis is crucial to the survival of the brain as there are series of redundancies which, upon vessel blockage, continue to allow blood circulation to prevent possible ischemia. Branching from the circle of Willis are blood vessels known as the anterior cerebral arteries, posterior communicating arteries, carotid termini, posterior cerebral arteries, and the apex of the basilar artery. Tiny critical arterial branches branch from the circle of Willis branch arteries and supply blood to the deep structures of the brain. All of the brain arterial blood eventually drain into the internal jugular vein in the neck and exit the brain traveling towards the heart[18-21].

Brain arteries function to deliver blood containing oxygen and nutrients, such as glucose, to the brain. At any given time, one-fifth of the heart's output is directed towards the brain. The brain consumed glucose as is substrate to eventually create ATP. In fact, 120 grams of the body's required 160 grams of sugar are required by the brain[22]. Veins complete the blood circuit by draining metabolic waste products from the brain. The flow of blood throughout the entire cranium involves all aspects of the blood vessels which includes the three layers of the blood vessels, nerve plexi, and metabolic processes of cells[23,24].

Anatomy of Brain Arterial Blood Vessels

Brain arteries contain many anatomical features which must be understood. First, there are 3 distinct layers of the artery that contain different types of cells. The layer in contact with the blood (luminal surface) is called the tunica intima which contains endothelial cells surrounded by the internal elastic media. The tunica intima is responsible for the detection of chemicals in the blood and proper responses (i.e. transduction/response to hormones). The middle layer is called the tunica media and primarily contains smooth muscle cells as well as collagen which is surrounded by the external elastic lamina. The tunica externa lays superficial to the external elastic lamina and can be referred to as the adventitial layer of the blood vessel. This layer contains a large amount of collagen which is used to anchor the artery to surrounding organs giving it rigidity[21].

Arterial Endothelial Cells

BBECs are different from extracranial endothelial cells. First, BBEC contain more mitochondria than other endothelial cells[25] which is supported by the fact that glucose oxidation is the primary, or only, source of ATP formation in the brain[22]. Also, Nox4 expression is about 10-fold higher in cranial endothelial cells versus extracranial cells[26,27]. Nox2 is expressed in extracranial endothelial cells and smooth muscle cells and not in cranial endothelial cells[26,27]. Different Nox protein expression will result in differences between cells due to the stimulus for the Nox proteins being different. Finally, cranial endothelial cells are less likely to develop atherosclerosis than extracranial endothelial cells[28]. Atherosclerosis is the main source of

abdominal aortic aneurysm formation and is typically only seen in brain arteries of aged individuals[28].

In response to chemical and physical stimuli, BBECs produce many blood vessel modulators (e.g. nitric oxide (NO) and hydrogen peroxide) which control the constriction and relaxation of the blood vessel[29]. For example, NO (previously known as endothelium-derived relaxation factor or EDRF) is produced by the endothelial Nitric Oxide Synthase (eNOS) complex and diffused to the smooth muscle cells (SMCs) of the tunica media and results in vasorelaxation[29,30].

My focus of this research is to investigate a possible reason why Diabetes Mellitus (DM) is such a large risk factor for the development of intracranial aneurysms (IA). IA formation typically occurs in flanking walls of bifurcated arteries within the circle of Willi or its primary branches due to early endothelial responses[31]. Meng et al found that early IA formation and damage was a result of ROS formation in endothelial cells which decrease smooth muscle cell tone in the tunica media via an apoptotic mechanism[31]. Therefore, I used bovine brain arterial endothelial cells (BBECs) as my *in vitro* model.

IA formation

IAs are outward projections of weakened blood vessels and affect approximately 1 in 20 people. Most of these IAs are not be detected as they mostly are non-problematic and asymptomatic. However, once symptoms arise, the IA has become a substantial threat to rupture. Approximately 30,000 citizens in America suffer from IA rupture which is also known as aneurysmal subarachnoid hemorrhage. Unfortunately, IA rupture is approximately 65% lethal. In fact, about 50% of ruptures result in the death of the patient before arrival to a hospital or

treatment facility. A large percentage of the survivors of rupture will live with permanent damage[21].

IAs typically form within bifurcations of the arteries in the circle of Willis and its primary branches where cells experience high flow conditions. For the purpose of my thesis, flow refers to the wall shear stress (WSS) exerted by the flow of blood on the endothelium. WSS is the frictional drag, or pressure, of the blood on the endothelium. Recent research has shown that IA formation is due to high flow through production of and successive destruction by radical/reactive oxygen species (ROS). Meng et al found that administration of antioxidants to rabbits whom had induced aneurysm development reduced early IA damage leading them to believe that early IA formation and damage was due to ROS. Further experimentation showed that ROS produced within an intact endothelium decreased the amount smooth muscle cells in the tunica media which decreased the strength of the artery resulting in IA development[31]. Therefore, initial IA development occurs due to endothelial dysfunction.

After endothelial dysfunction, a mounting inflammatory response occurs which results in high levels of cytokine. A specific modulator of inflammation is the transcription factor NF-κB where the κB portion translocates to the nucleus and upregulates cytokine gene expression under ROS production and flow transduction. This, in turn, changes the phenotype of the surrounding smooth muscle cells which activates matrix metalloproteinase activity. Matrix metalloproteinases (MMPs) are responsible for the degradation of the extracellular matrix of endothelial cells and smooth muscle cells. Upon MMP activation, vascular remodeling acts to excise the affected region of dysfunctioning cells. During vascular remodeling and MMP activity, collagen biosynthesis in smooth muscle cells decrease as apoptosis rates increase decreasing the tone of the tunica media creating a weakening of the artery. Then, an IA can completely form[32].

ROS

ROS are oxygen species with unbalanced electrons in its valence shell. The more commonly known ROS are superoxide, hydrogen peroxide, nitric oxide (NO), and peroxynitrite. NO and hydrogen peroxide are better known for their modulation of the tone of blood vessels[33]. In brain arteries, NO production in the endothelium results in relaxation of the smooth muscle cells in the tunica media and successive vasodilation. This typically occurs in response to stimuli in the blood such as occlusion of another vessel, changes in blood chemistry, and high flow.

How do ROS form? First, it is important to note that the electronic configuration of the valence shell of molecular oxygen prevents it from accepting more than one electron at a time. Oxidase activity within endothelial cells transfer electrons from a substrate to molecular oxygen creating superoxide. Superoxide then can either bind to nitric oxide to create another species of ROS (peroxynitrite), undergo enzymatic activity by superoxide dismutase (SOD) to become hydrogen peroxide, or react whether it be in or out of the cell[33].

Superoxide is of great importance when discussing oxidative stress. It can be created by NADPH oxidase, uncoupled endothelial nitric oxide synthase (eNOS), xanthine oxidase, or oxidases in the mitochondrial electron transport chain (ETC) which will all be discussed shortly. Superoxide can then react with various intracellular or extracellular proteins and modify their behavior. One of the more important proteins targeted is ferritin which is a protein that is responsible for iron storage increasing free iron within the cell. Iron can further react with superoxide to create one of the most potent oxidant in nature, the hydroxyl radical. The hydroxyl radical which causes DNA mutations, DNA strand breaks, lipid peroxidation, and protein modification which may either lead to the toxicity of the cell or the death of the cell through

apoptosis[33]. For this reason, superoxide is a very important molecule in the pathogenesis of IA formation.

Diabetes Mellitus

Diabetes Mellitus (DM) is a significant health concern in the United States affecting at least 25.8 million people which is roughly 8.3% of the total population. It is a health risk for micro- and macrovascular diseases, specifically IA formation[1]. DM patients experience elevated resting blood glucose levels when compared to healthy patients which is an important characteristic of the disease to note[1].

High glucose has been shown to cause increases in ROS production in rats[1,2], human umbilical vein endothelial cells[8], bovine aortic endothelial cells[34], and humans undergoing arterial bypass surgery[35]. A large portion of evidence supports the mitochondrial electron transport chain as the main source of ROS production due to high glucose[33,36-40]. Briefly, superoxide can be generated at complex I, III, and IV of the mitochondrial ETC due to incomplete transfer of electrons to molecular oxygen[33].

My research focuses on the effect of high glucose on ROS production in BBECs which is intended to simulate the resting blood glucose concentration of DM patients. Since DM is a prominent and destructive disease, I wanted to research the effect of glucose on ROS production, which has been shown to play a very important role in IA development.

DM and IA Formation

Aside from DM increasing ROS production in endothelial cells, IA formation is also stimulated by high glucose. A rather substantial amount of evidence suggests that DM enhances

matrix metalloproteinase activity which degrades the extracellular matrix of endothelial cells and smooth muscle cells causing a further weakening of the vessel wall[34]. While matrix metalloproteinase activity is activated under high glucose, a lot of DM effects on IA formation boil down to one major causative agent, ROS. Therefore, it is important to understand what ROS normally do in endothelial cells and how they behave in the pathological model such as IA formation.

Positive Effects of ROS

Low levels of ROS are needed by cells and systems to perform normal physiological functions. ROS are used as a signaling molecule for proliferation, increased cell membrane permeability, cell growth, and apoptosis[3,38]. Normally, apoptosis is thought to be a negative mechanism, but ROS can, in normal models, initiate apoptosis due to cell damage (DNA mutations, dysfunction, or control of cancer development). ROS may also play a role in the modulation of vascular tone. For example, NO and hydrogen peroxide can cause relaxation of the smooth muscle cells in the tunica media resulting in vasodilation due to a specific stimulus such as high flow. Finally, ROS play an important role in angiogenesis after a cardiovascular injury by stimulating growth and proliferation of endothelial cells[3,38,41].

Negative Effects of ROS

When ROS levels elevate beyond baseline levels, they can become dangerous to the integrity of the cell for a couple of reasons. First, elevated ROS levels interact with NO decreasing the bioavailability of NO for vasorelaxation[41]. Even though another radical is formed, the loss of available NO can become detrimental to the integrity of the blood vessel as its ability to respond to stimuli, such as high flow, is augmented. Second, elevated ROS initiate pro-

inflammatory responses which result in the accumulation of vascular cell adhesion molecules and intracellular adhesion molecules which result in local inflammation of the affected region[41]. Inflammation initiates ROS production in adjacent cells resulting in a domino effect of ROS destruction.

ROS also can interact with cellular DNA and cause strand breaks which activate poly(ADP) ribose polymerase activity which repairs strand breaks[36,42]. Peroxynitrite is a potent initiator molecule of single-strand DNA breaks and is created after the reaction of superoxide with NO[36,42]. Poly(ADP) ribose polymerase stimulates cytokine and adhesion molecule production resulting in a local inflammatory response[36,42].

ROS have the ability to react with proteins and modify their function. For example, ROS may interact with ferritin, an intracellular iron storage protein, causing an increase in free iron. An interaction between a transition metal, such as iron, and superoxide produces one of the most potent ROS in nature. The hydroxyl radical is highly reactive and extremely destructive[33]. Cells experiencing oxidative stress due to hydroxyl radical production will most likely undergo apoptosis to prevent further production of the radical.

The danger of DM can now be seen as elevated ROS results in a wide variety of negative effects in the cell. One can begin to connect how high glucose, which increases ROS production, is a potent risk factor for many macrovascular diseases, such as IA formation.

Sites of Production in Endothelial Cells

NADPH Oxidase

The NADPH oxidase complex is a very large ROS producing complex within cells, specifically endothelial cells[9,26,35,36]. The complex consists of 2 membrane components, a Nox protein and $p22^{phox}$, as well as 4 cytosolic subunits (Rac, $p47^{phox}$, $p67^{phox}$, and $p40^{pho)}$ which must be bound in the complex in order for the complex to be active[26].There are 5 Nox subunits which have differential expression throughout the body (i.e. only certain areas of the body express 1 or 2 Nox subunits)[26].

Nox is the key catalytic component of the NADPH oxidase complex which have Flavin-binding regions[9]. Upon binding to the subunit, NADPH serves as the electron donor to molecular oxygen which then is converted into superoxide. Restoration of $NADP^+$ is essential to the cell as it serves as an electron acceptor to cellular processes including energy production.

Since NADPH oxidase produces superoxide, it is a very important protein to endothelial cell physiology and pathophysiology. As described previously, superoxide can act as a signaling molecule for growth, proliferation, cell membrane permeability, and NO sequestering. However, the negative effects of ROS are more important when discussing the pathology of macrovascular pathologies. Inflammation within the cell, typically seen during IA development, stimulates the NADPH oxidase complex directly which further increases ROS production[9]. Inhibition of the NADPH oxidase complex using diphenyleneiodonium (DPI) significantly decreased ROS production in HUVECs[8].

In DM patients, high glucose is believed to stimulate NADPH oxidase as a downstream effect of the activation of phosphokinase C (PKC)[8,9]. PKC activation is ROS-independent so high glucose has been hypothesized to be the molecule responsible for PKC activation and

downstream NADPH oxidase activation[8,35,36]. PKC inhibition decreased the activation of NADPH oxidase in HUVECs treated with high glucose[8].

NADPH oxidase is a very important contributor to the ROS production within endothelial cells. High glucose, indirectly, can increase NADPH oxidase activity which poses yet another threat to DM patients in the development of macrovascular complications due to their disease.

Uncoupled Endothelial Nitric Oxide Synthase

Endothelial nitric oxide synthase (eNOS) is a very important modulator of vascular tone. L-Arginine, molecular oxygen, NADPH, and tetrahydrobiopterin enter the enzyme and result in the production of citrulline and NO[5,29,43]. Normal eNOS function transfers electrons from tetrahydrobiopterin and NADPH oxidase to L-arginine to produce L-citrulline[5,29]. In brain arteries, NO causes vasodilation in response to a chemical or physical stimulus[5,29,43]. NO bioavailability is decreased under oxidative stress as available NO reacts with superoxide to create peroxynitrite instead of causing vasodilation[5,29].

Consequently, eNOS may contribute to the generation of ROS in response of oxidative stress. Pre-existing ROS oxidize tetrahydrobiopterin to produce dihydrobiopterin[5,43]. Dihydrobiopterin does not have donor electrons available and is unable to transfer any electrons to L-Arginine. Electrons from NADPH are transferred to molecular oxygen instead of L-Arginine creating superoxide which can react with more tetrahydrobiopterin, decrease the bioavailability of NO, react with proteins such as ferritin and DNA, or cause inflammation and apoptosis.

High glucose contributes to increased ROS production by uncoupling eNOS. Hink et al found that ROS levels within the cells was increased coming from uncoupled eNOS[2]. The

process of eNOS uncoupling occurs after early oxidation of tetrahydrobiopterin contributing to further oxidative stress.

Xanthine Oxidase

Xanthine oxidase (XO) is an enzyme which catalyzes the oxidation of hypoxanthine and xanthine in the process of purine metabolism by reducing molecular oxygen to produce superoxide[29]. Purine metabolism is essential for cellular function as it modulates proliferation by decreasing purine availability for DNA replication. XO may be present in either its XO form or its xanthine dehydrogenase form and can be converted from its dehydrogenase form to its oxidase form by oxidation of critical cysteines[44]. Activated NADPH oxidase is essential for the functionality of XO. McNally et al found that cells lacking the NADPH oxidase regulatory subunit, $p67^{phox}$, have a marked reduction of XO expression[44].

ROS derived from XO have been shown to decrease the bioavailability of NO[29,41,44]. In diabetic rats, inhibition of XO by oxypurinol improved vasodilation, which was previously impaired, meaning that there was a rescue in NO bioavailability[29].

Mitochondrial Electron Transport Chain

The mitochondrial electron transport chain (ETC) produces ROS constitutively as a result of incomplete electron shuffling to oxygen. The electron configuration of the valence shell of molecular oxygen prevents it from accepting more than one electron at a time meaning that the complete reduction of oxygen to water must happen in multiple monovalent steps. This opens the possibility for incomplete reduction of oxygen resulting in ROS generation. Under aerobic respiration, reduced co-enzymes produced through the oxidation of glucose enter the mitochondrial ETC to be oxidized and regenerated for future glucose oxidation.

ROS can be generated at multiple sites of the mitochondria ETC. First, NADH dehydrogenase, or complex I, and succinate dehydrogenase, or complex II, are believed to produce ROS as a result of one of its subunits (N1a) resulting in pooling of superoxide within the core of the enzyme. Under complex I and/or II inhibition, it is believed that these pools are released causing an increased presence of ROS within the mitochondria as well as the cytoplasm[33]. Second, cytochrome c oxidase, or complex IV, may produce ROS as electron transfer between complexes can result in electron leakage[3]. Also, this is the location in which molecular oxygen is reduced to water[3,33]. Thus, electrons can only be added to the valence of molecular oxygen one at a time resulting in four monovalent additions. Under normal physiological conditions, incomplete reduction of molecular oxygen occurs at a rate of 5% leading to the formation of superoxide and hydrogen peroxide at this complex[33]. Since electrons may leak from the transfer between cytochrome c to complex IV, ROS can be generated in both the mitochondrial matrix and the intramembranous space[3,33]. Inhibition of complex IV by cyanide or related compounds decreases its reduction potential preventing the transfer of electrons. Thus, electron transfer is prevented throughout the entire chain resulting in the inhibition of the entire chain[3,33].

High glucose increases the activity of the mitochondrial ETC resulting in a higher production of ROS[3,33,36]. More glucose is oxidized by the TCA cycle which produces more electron donors to be used in the ETC. This causes the voltage gradient across the mitochondrial membrane to increase as it approaches a critical threshold in which electron transfer is blocked. This causes electrons to pool in coenzyme Q which donates one electron at a time to molecular oxygen producing superoxide[36]. Brownlee et al found that administration of MnSOD to cells experiencing hyperglycemia reduces ROS production[36]. MnSOD is an enzyme which is unique

to the mitochondria as it dismutates superoxide to form hydrogen peroxide. Hydrogen peroxide may then be converted to water via catalase.

The mitochondria can also be mechanotransducers of flow stimulation[33]. The cytoskeleton rearranges its conformation as a result of flow stimulation. The mitochondria are directly associated with the cytoskeleton which can help explain its mechanotransducing properties. The mechanical force can be transmitted through the microtubules to the mitochondria. Mechanotransduction of the signal must then be converted into biological signals which may occur via the Na^+/H^+, Na^+/Ca^{2+}, or K^+/H^+ exchangers but the mechanisms are not well understood[33].

This research aims to investigate the effects of glucose on flow-induced ROS production. By understanding this *in vitro*, we may be able to characterize mechanisms to investigate *in vivo* with a goal of developing therapies to prevent DM complications.

Methods

Cell Culture

Frozen stock cultures of Bovine Brain Endothelial Cells (BBECs) were provided by Joseph Moran-Guiati in our laboratory. Briefly, Joe obtained brain arterial endothelial cells from the brains of freshly decapitated steer from a local slaughterhouse. The specific region of sampling was within the circle of Willis and its primary branches. Upon identification of the circle of Willis and its primary branches, arteries were excised and cut into segments under 4 cm and washed with sterile PBS. Segments were then transferred into a petri dish containing Dulbecco's modified Eagles medium (DMEM, Life Technologies, Inc., Grand Island, NY) with 10% fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA) and an antibiotic solution (100 U/ml penicillin, 100 mg/mL streptomycin; Life Technologies Inc., Grand Island, NY). Vessels

were split longitudinally and the luminal surface, which contains the endothelium, was gently scraped with a scalpel. The scrapings were placed in petri dishes containing DMEM in 10% FBS and the antibiotic solution. The cells were allowed to settle, attach, and grow until small colonies were visible. The samples were incubated at 37°C in 5% CO_2.

To subculture clonal populations, cells from single colonies conforming to a cobblestone appearance were separated by a glass-cloning ring. The medium within the ring was aspirated out and replaced with PBS. The PBS was then aspirated and the colony was incubated in 0.25% trypsin with 2.21 mM EDTA for 3 minutes until cells detached from the dish. After incubation, the solution containing the dissociated colony was aspirated and transferred to a tube containing equal amounts of DMEM and centrifuged. The pellet was re-suspended and transferred to a 25-cm^2 culture dish (BD Falcon, Bedford, MA) for further expansion. When cultured reached confluence, they were passaged to 75-cm^2 dishes and subcultured 2 times at 1:4 splits before stock cultures were aliquoted and cryopreserved.

Cell cultures were confirmed to be endothelial by testing for the expression of Factor VIII, Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1), endothelial Nitric Oxide Synthase (eNOS), and for LDL uptake. The LDL product used to measure for uptake was 1,1-dioctadecyl–3,3,3’,3’-tetramethyl-indocarbocyanine perchlorate (DiI) conjugated to a low density lipoprotein to form Dil-Ac-LDL (Biomedical Technologies). This complex was excited at a wavelength of 485 nm and emitted at 535 nm so that its transport into endothelial cells may be traced. All cells stained positively for Factor VIII, PECAM-1, and eNOS by immunofluorescence and took up Dil-Ac-LDL. In addition, all cells were negative for smooth muscle actin.

Thawed cultures of cells were grown in DMEM containing 5 mM glucose or 30 mM glucose. Cells were incubated at 37°C in 5% CO_2. Cells between passages 6 and 9 were used for experimentation.

Exposure of Cell to Flow

BBEC were seeded into 6 well flow chambers (IBIDI, Verona, WI) at a density of 125,000 cells per well. To subject endothelial cells to flow, peristaltic pumps were utilized to pump medium through the IBIDI chamber lanes. In order to more accurately simulate physiological hemodynamics (i.e. physiologically realistic normal wall shear stress and high wall shear stress levels at reasonable pump speeds), methylcellulose was included in the medium to raise the viscosity to around 5 milliPascal seconds (mPas), roughly the viscosity of bovine blood, without changing the osmolarity. Briefly, a 2% methylcellulose was created mixing methylcellulose (Sigma cat. No. M6385) with cold water. The mixture was autoclaved to sterilize the contents. After autoclaving, the sterile mixture was mixed for 24 hours at 4°C. After 24 hours, an equal volume of cold 2X DMEM (Life Technologies) was added to the solution and stirred for 24 hours at 4°C. After 24 hours, the viscosity was measured using a Cannon-Manning Semi-Micro Viscometer (Cannon Instrument Co.; State College, PA) at 40°C.

A flow apparatus was used to administer flow to confluent endothelial cells in IBIDI 6 well chambers. First, an autoclaved medium reservoir was filled with viscous DMEM warmed to 37°C and equilibrated to 5% CO_2. Sterile tubing connected the medium reservoir to a Peristaltic Pump P-3 (Pharmacia Fine Chemicals, NYC, NY) which was then connected to one lane in the flow chamber by more sterile tubing. The outlet was connected by more sterile tubing back to the medium reservoir to complete the flow system. To achieve high flow, a Minipuls 2 pump

(Gilson, Inc, Middleton, WI) was used which was able to pump viscous medium through the flow chamber at a higher speed.

Wall shear stress (WSS) experienced by cells in the flow chamber was calculated based on the Navier-Stokes equation with the following formula:

$$WSS = \frac{(6)\times(\text{viscosity of medium})\times(\text{flow rate})}{(\text{width if chamber})\times(\text{height of chamber})^2}$$

Flow rates were adjusted (by adjusting pump speeds) to obtain two different shear stresses. Intracranial aneurysm development occurs at specific bifurcations within the circle of Willis and its primary branches[11,20,45,46]. At these bifurcations, a large shear stress typically measured to be greater than 8 Pa, but it was not extraordinary to observe shear stresses well over 30 Pa[11,45,46]. Alfano et al found that the location in which IAs transition between rare occurrences to relatively common occurrences takes place between 5 Pa and 15 Pa[20]. Such regions experiencing these shear stresses occur at the apices of bifurcations of blood vessels within the circle of Willis and its primary branches[11,20,45,46] leading me to use a shear stress of 10 Pa. Likewise, baseline wall shear stress has been found to be between 1.5 Pa and 2.5 Pa in straight blood vessels[20,47]. Therefore, I used a flow rate that produced a WSS of 2.0 Pa as my "normal flow" condition.

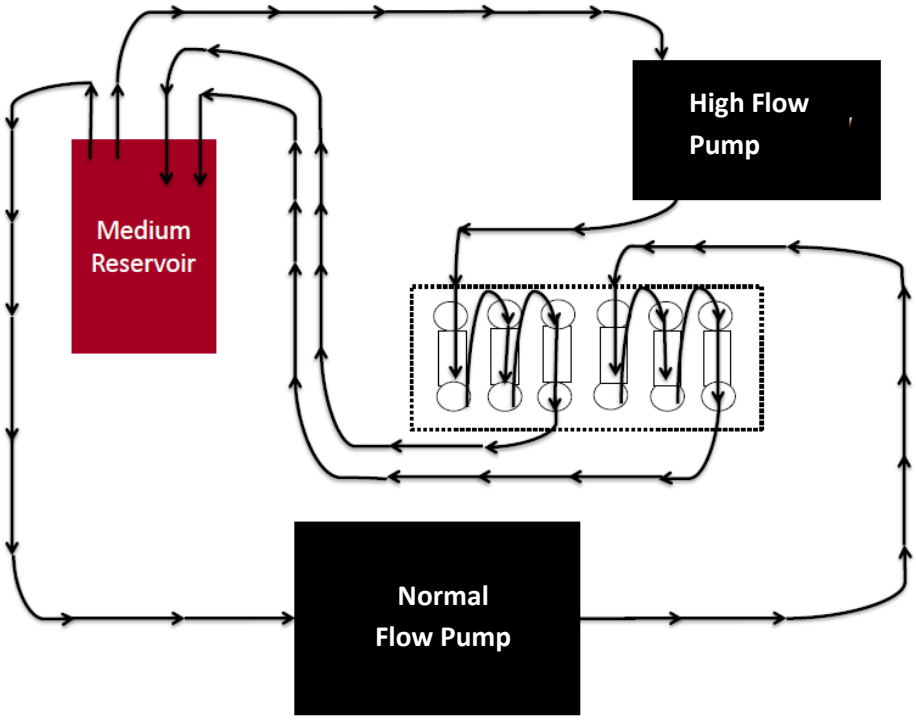

Figure 1: Schematic representation of flow apparatus. The diagram above shows a typical flow set-up in which endothelial cells are subjected to flow using sterile tubing. Medium in pumped via sterile tubing (black lines) in the directions indicated with the arrowhead from the medium reservoir (red box) through either the normal flow pump or the high flow pump to the flow chamber. At the flow chamber, the medium enters at the top of the plate and is pumped in the direction indicated by the arrowheads. Sterile tubing (black lines) connects the bottom of the first flow lane to the top of the second flow lane and medium is pumped from the top of the lane to the bottom of the lane. Sterile tubing (black lines) connects the bottom of the second flow lane to the top of the third flow lane and medium is pumped from the top of the lane to the bottom of the lane. Medium then flow back to the reservoir. This arrangement occurs separately for the high flow pump and the normal flow pump.

ROS Detection

A total of 4 different assays were used to measure ROS.

Nitro Blue Tetrazolium Assay. This assay was used to measure ROS in static culture. Cells were seeded in 24-well plates at a density of 100,000 cells per well and incubated for approximately 24 hours. Then, DMEM was removed and a solution containing 2 mg/mL Nitro

Blue Tetrazolium (Life Technologies) and 5 μM Hoechst dye (Life Technologies, Inc.) was added to each well. The assay was incubated in the dark at 37°C in 5% CO_2 for 90 minutes. Each well was washed 4 times with sterile PBS containing 2 mM $MgCl_2$ and 1 mM $CaCl_2$. Hoechst fluorescence was read on intact live cells while in the final PBS wash using a Biotek Plate Reader measuring an emission of 360 nm after excitation at 460 nm. The fluorescence was used to quantify the relative number of cells in each well based on Hoechst staining of DNA. PBS was then removed from all wells and a lysis solution containing 90% DMSO, ddH_2O, 0.1% SDS, and 0.01 N NaOH was added which lysed cells and solubilized formazan (reduced NBT). Absorbance of the solution was measured at 700 nm. The absorbance measured the amount of NBT reduced by ROS within the cells. Upon reduction, NBT precipitated in aqueous solution.

Dihydroethidium (DHE) Assay. To measure ROS in cultures exposed to flow, fluorescent ROS indicators, dihydroethidium (DHE) or CellROX, were used instead of NBT because the amount of material in the flow channels was too small to measure in the NBT assay. Upon interaction with ROS, DHE becomes oxidized to ethidium which intercalates into DNA and fluoresces under an excitation wavelength of 510 nm at an emission wavelength of 615 nm[48]. Cells were treated with 5 μM DHE in DMSO (Molecular Probes, Eugene, OR) for 30 minutes, then washed 3 times using PBS and imaged live using a Zeiss Axiovert 135 fluorescence microscope at magnification 20X under with an excitation wavelength of 510 nm and emission was imaged with 590 nm long pass filter[49].

Approximately 10 fields in the middle of each lane were imaged using a Zeiss Axiovert 135 Fluorescence Microscope at 20X and fluorescence was quantified using ImageJ software[50]. Cells were manually counted. Background fluorescence was established by measuring the average integrated density of regions devoid of cells for each image. This value was subtracted

from the entire image field and integrated density of the entire field was then measured. Integrated density per cell was calculated by dividing the integrated density for each image by the cell count for that image.

CellROX Assay. CellROX, like DHE, is oxidized by ROS and then intercalates in the DNA and fluoresces. CellROX fluorescence was imaged using an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Cells treated with CellROX can be fixed with 3.7% formaldehyde to stop continued reactivity within the cell without losing fluorescence of the reacted CellROX. Cells were treated with 5 μM CellROX (Molecular Probes, Eugene, OR) for 30 minutes, washed 3 times using PBS, and fixed in a 3.7% formaldehyde solution in PBS at room temperature for 5 minutes. Cells were then imaged using a Zeiss Axiovert 135 fluorescence microscope at magnification 20X using and excitation wavelength of 485 nm and an emission wavelength of 520 nm[49].

MitoSOX Assay. In order to measure mitochondria-specific ROS, an ROS probe that localized to mitochondria (MitoSOX Red) was used. MitoSOX Red is a cell permeant molecule that localizes to the mitochondrial membrane[51]. When superoxide oxidizes MitoSOX, it becomes fluorescent when excited at 510 nm, fluorescing at 615 nm[48,49]. Cells were treated with 5 μM MitoSox Red (Life Technologies, Inc.) and incubated at 37°C in 5% CO_2 for 10 minutes in DMEM and washed three times with warm PBS containing 2 mM $MgCl_2$ and 1 mM $CaCl_2$. However, cells treated with MitoSOX were not fixed because the fluorescence and localization are not retained. Cells were imaged live using a Zeiss Axiovert 135 fluorescence microscope at magnification 20X with an excitation wavelength of 510 nm and emission was imaged with 590 nm long pass filter.

Statistical Analysis

Results are expressed as means ± SEM. Statistical comparisons between groups were assessed by Student's t-test. A p-value of $p<0.05$ was considered to be significant.

Results

BBECs produce more ROS in high glucose

To determine the effect of high glucose on ROS production by brain arterial endothelial cells, BBECs were incubated in DMEM containing either 5 mM or 30 mM glucose and then an NBT assay was performed to measure ROS production. 5 mM glucose represents the normal fasting blood glucose concentration whereas 30 mM glucose represents a resting blood glucose concentration in uncontrolled diabetes.

Figure 2 shows that treatment of BBECs with 30 mM glucose for 90 minutes increased ROS production 2.5-fold relative to BBECs cultured in 5 mM glucose. 24-hour exposure to high glucose increased ROS production an additional 1.5-fold. Returning cells to 5 mM glucose after long-term treatment with 30 mM glucose resulted in a nearly complete return to baseline ROS production within 90 minutes.

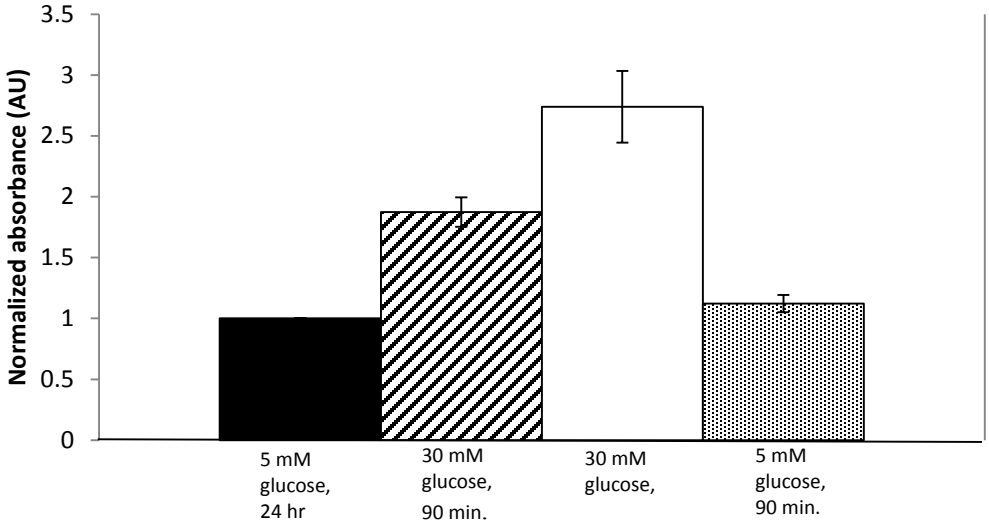

Figure 2: Effect of high glucose on ROS production. BBECs were exposed to 5 mM or 30 mM glucose for the indicated times and ROS measured by NBT reduction. Each bar represents the mean of 3 experiments. Data was normalized to the average absorbance of endothelial cells grown in normal glucose for 24 hours. Bars represent ± the standard error of the mean. There is a statistically significant difference between baseline ROS production and short-term glucose treatment ($p<0.001$).

High glucose caused increased ROS production from the mitochondrial electron transport chain

To determine if high glucose increases ROS production because of increased activity in the mitochondrial electron transport chain, mitochondrial oxidative phosphorylation was inhibited using the complex IV inhibitor, sodium azide. Cells were grown in either 5 mM glucose or 30 mM glucose for 24 hours in 24 well plates. An NBT assay was performed to measure ROS production by BBEC while the mitochondrial electron transport chain was inhibited using sodium azide. Schwoebel et al found that 10 mM azide was practical concentration needed to successfully inhibit the mitochondrial ETC[52]. I performed a titration that confirmed that 10 mM sodium azide was an appropriate concentration to use in order to inhibit the mitochondrial electron transport chain (data not shown). Figure 3 shows that sodium azide inhibited ROS production of BBECs in high glucose by 49% ($p<0.05$).

To test mitochondrial-specific ROS production, static cultures treated with 5 mM glucose or 30 mM glucose were treated with 10 mM sodium azide for 15 minutes and stained with MitoSOX Red. Figure 4 shows that mitochondrial ROS was innately elevated in high glucose-treated cultures versus normal glucose-treated cells. Inhibition of the mitochondrial electron transport chain decreased ROS production 36% in high glucose ($p<0.05$) whereas the mitochondrial electron transport chain was inhibited 26% in normal glucose. The amount of ROS production in azide-treated high glucose cultures mimicked the ROS production in non-inhibited normal glucose-treated cultures.

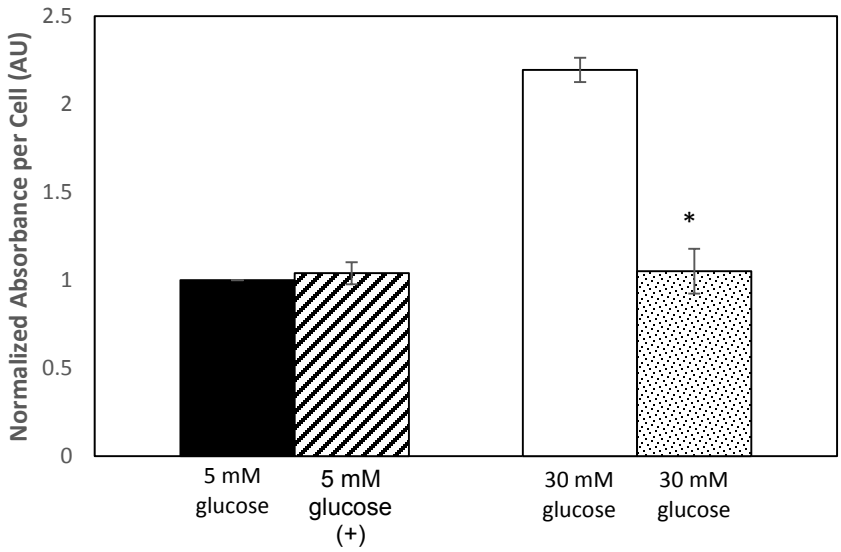

Figure 3: Effect of azide on ROS production. 10 mM **s**odium azide was used to inhibit the mitochondrial electron transport chain and an NBT assay was used to measure ROS. ROS is expressed as NBT absorbance per cell based on Hoechst staining. Each bar represents mean ± the SEM of 3 experiments. *Significance was found between un-inhibited high glucose-treated BBEC and inhibited high glucose-treated BBEC ($p<0.05$).

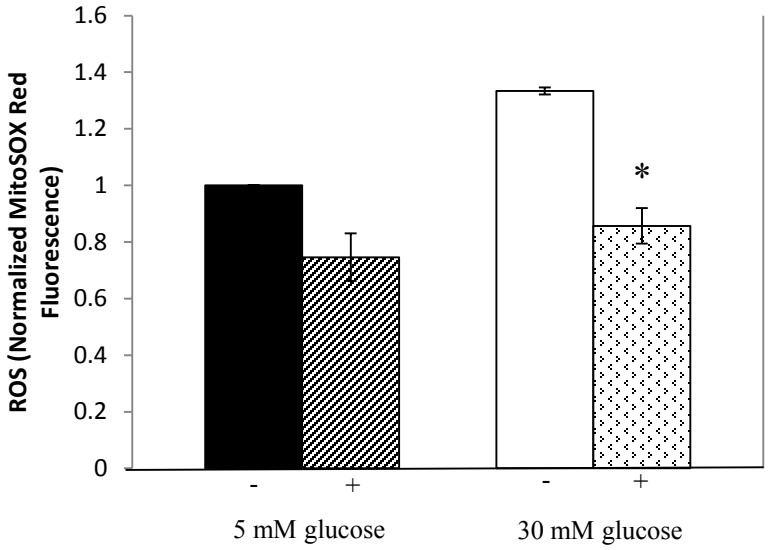

Figure 4: Effect of azide on ROS production using MitoSOX Red. 10 mM sodium azide was used to inhibit the mitochondrial electron transport chain and a MitoSOX Red assay was used to measure ROS. ROS is expressed as MitoSOX Red fluorescence per cell normalized to the average MitoSOX Red fluorescence of BBECs treated with normal glucose. The results are represented as mean normalized fluorescence ± the SEM of 3 experiments. * Significance was found between un-inhibited high glucose-treated BBEC and inhibited high glucose-treated BBEC ($p<0.05$). ROS in azide-treated cells was not significantly different from untreated cells in 5 mM glucose ($p>0.05$).

To determine the contribution of NADPH oxidase, xanthine oxidase, and eNOS to the production of ROS due to glucose, each complex was inhibited separately and ROS levels were measured using an NBT assay with Hoechst staining. Inhibition of NADPH oxidase was performed using 20 μM diphenyleneiodonium (DPI). Endothelial NOS was inhibited using 100 μM L-Nitro-Arginine Methyl Ester (L-NAME). Xanthine oxidase was inhibited using 50 μM Febuxostat. Inhibitions were done in BBEC treated with either 5 mM or 30 mM glucose. NBT was added simultaneously with the inhibitor.

Figure 5 shows that eNOS and XO did not contribute to ROS production in BBEC. In fact, inhibition of these enzymes caused ROS to increase ($p>0.05$). DPI caused a relatively large inhibition of ROS production in high and normal glucose ($p<0.05$). The average inhibition of DPI in 5 mM glucose was 50% versus 62% in 30 mM glucose. ROS production was not due to a

difference in osmolarity as cells treated with medium containing 25 mM mannitol and 5 mM glucose showed no significant difference between normal glucose-treated cells and mannitol-treated cells

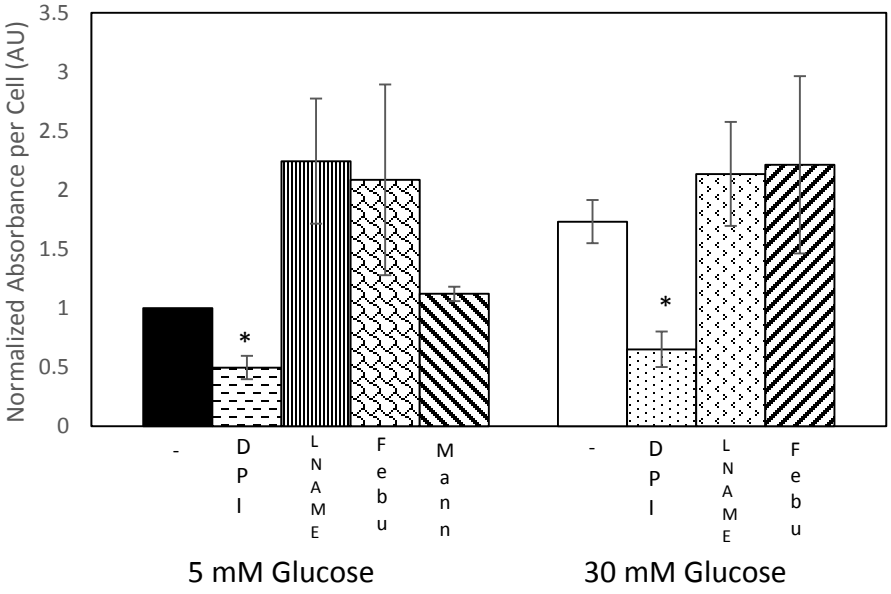

Figure 5: Effect of DPI, L-NAME, and Febuxostat on high and normal glucose-stimulated ROS production. 20 μM DPI, 100 μM L-NAME, and 50 μM Febuxostat were used to inhibit NADPH oxidase, eNOS, and Xanthine Oxidase, respectively, and an NBT assay was used to measure ROS. Mannitol was used as an osmotic control. ROS is expressed as NBT absorbance per cell based on Hoechst staining. The results are represented as mean absorbance ± the SEM of 3 experiments. *Significance was found between non-inhibited BBEC in high glucose and DPI-inhibited BBEC in high glucose as well as between non-inhibited BBEC in normal glucose and DPI-inhibited BBEC in normal glucose ($p<0.05$). There was no statistical significance between DPI-inhibited BBEC in normal glucose and DPI-inhibited BBEC in high glucose ($p>0.05$).

Effects of flow on ROS production

To determine if high flow increases ROS production, cells were exposed for 24 hours to flow at different rates and ROS production was measured at the end using DHE. The rate of flow was adjusted to obtain either a shear stress of 2 Pa or 10 Pa. 2 Pa was used to simulate baseline physiological wall shear stress levels[53] while 10 Pa was used to simulate high wall shear stress[20].

Figure 6 shows that cells subjected to normal flow produced 40% more ROS than static cells ($p>0.05$) and approximately 2.75-fold more ROS under high flow ($p<0.05$).

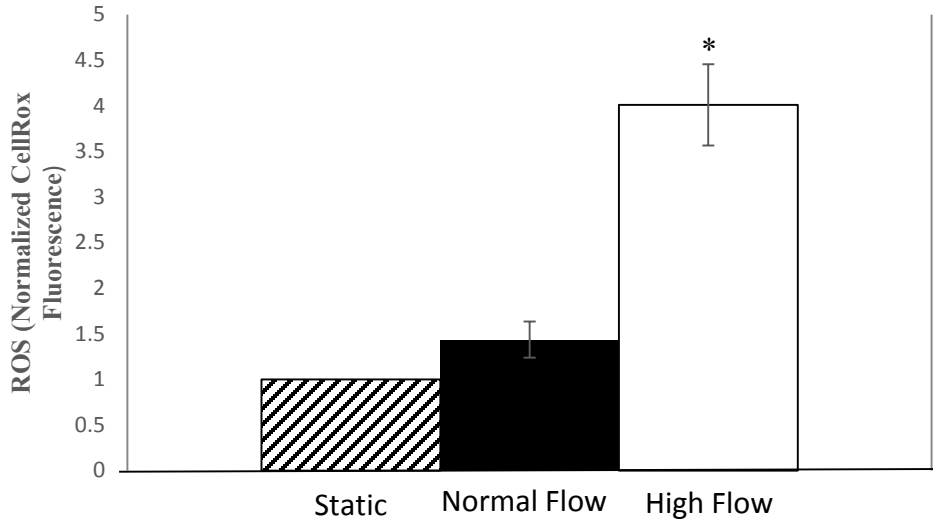

Figure 6: Effects of flow on ROS production. Cells were treated with either no flow, normal flow, or high flow for 24 hours and treated with DHE for 30 minutes. Values were normalized to the average integrated density per cell for the static condition. There was no statistical significance between the ROS production in normal flow versus static culture. *High flow was significantly different from normal flow and static ($p<0.05$).

High glucose increases ROS production in response to flow

To determine if glucose sensitizes BBEC response to flow-induced ROS production, cells were subjected to high or normal flow for 24 hours in either high or normal glucose. ROS production was then detected using CellROX at the end of the experiment. In normal glucose, high flow increased ROS production by 60%, whereas in high glucose, high flow increased it by 350% (Figure 7). From this, we can see that high glucose sensitized BBECs response to high flow.

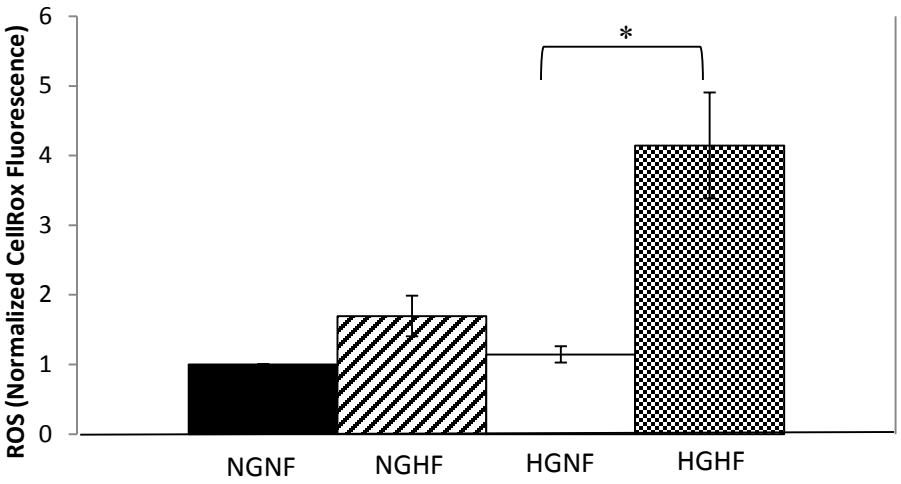

Figure 7: Effects of glucose on flow-induced ROS production. BBECs were treated with either high or normal flow in either high glucose or normal glucose for 24 hours. ROS was measured using CellROX after the flow exposure. To compare multiple experiments, values were normalized to the integrated density per cell of an NGNF sample run in each experiment. Bars represent mean ± the standard error of the mean for 3 experiments. *Significance was found between NGHF and HGHF as well as HGHF and HGNF ($p<0.05$).

Mitochondrial Electron Transport Chain Contributes to Flow-Induced ROS Production in High Glucose

To determine if the mitochondrial ETC plays a major role in the production of ROS under HGHF, cells were plated in either high glucose or normal glucose and subjected to either high or normal flow for 24 hours. ROS was then measured using CellROX in the presence or absence of 10 mM sodium azide to inhibit the mitochondrial electron transport chain. Figure 8 shows that azide inhibited most of the high flow-induced ROS production in high glucose. There was no significant inhibition of flow-induced ROS production when cells in normal glucose were treated with 10 mM sodium azide.

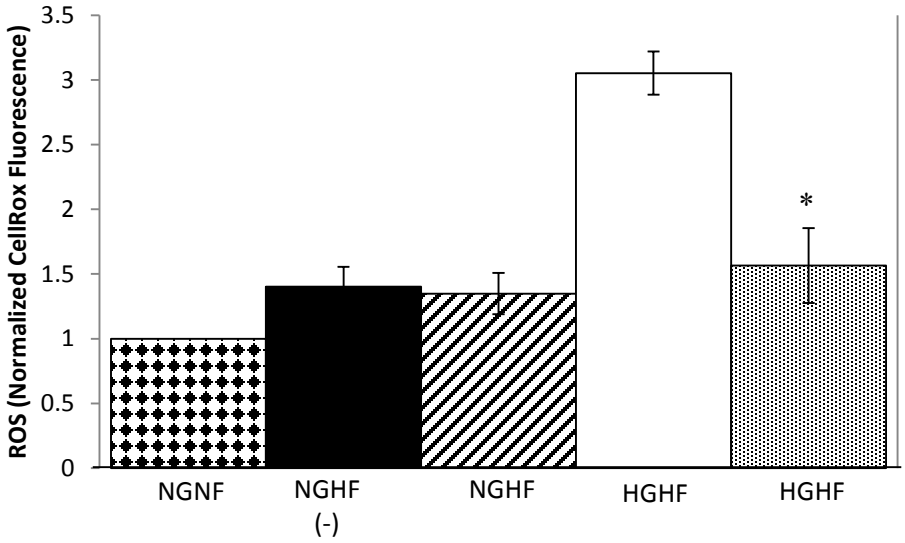

Figure 8: Azide attenuated high flow-induced ROS in high glucose. BBECs were subjected to either high or normal glucose under high flow and the ROS was detected using CellROX. The data is normalized to the averaged integrated density of the NGNF treatment. Statistically significant difference between the azide treatment in HGHF-treated BBEC and the un-inhibited HGHF-treated BBECs ($p<0.05$).

NADPH Oxidase does not significantly contribute to flow-induced ROS production in high glucose

To determine if the NADPH oxidase complex plays a role in the production of ROS under high glucose and high flow, cells were plated in either high glucose or normal glucose and subjected to either high or normal flow for 24 hours. ROS was measured using CellROX while the NADPH oxidase complex was being inhibited with 20 μM DPI. Figure 9 shows that in high glucose, DPI treatment inhibited approximately 10.2% of the flow-induced ROS production ($p>0.05$) whereas flow-induced ROS production in BBEC treated with normal glucose and subjected to high flow decreased 4.6% upon treatment with 20 μM DPI.

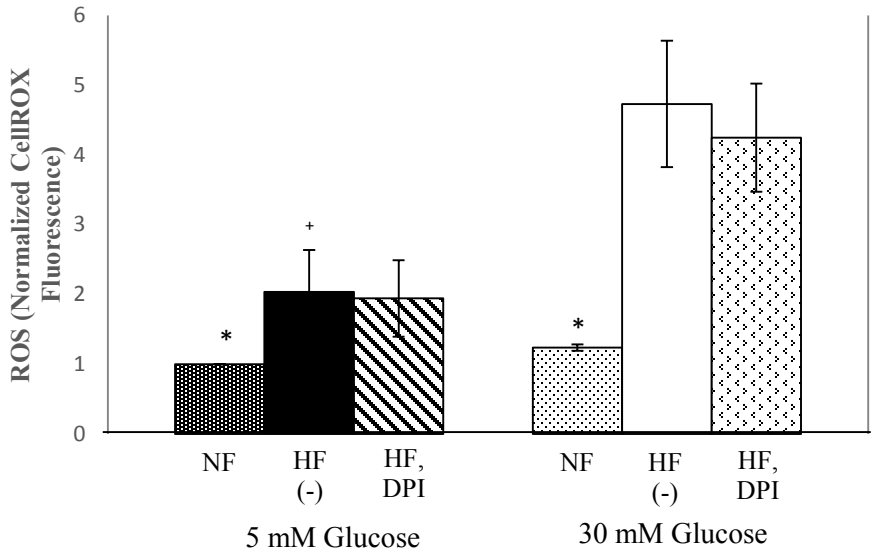

Figure 9: DPI had no effect on high glucose-induced ROS in high flow. BBECs were subjected to either high or normal glucose under high flow and the ROS was detected using CellROX. The data is normalized to the averaged integrated density of the NGNF treatment. *Statistical significance was found between high and normal flow in both high glucose-treated and normal glucose-treated BBEC ($p<0.05$). $^{+}$Significance was found between high flow-treatments of BBEC in high glucose and normal glucose ($p<0.05$).

DISCUSSION

My results show that high extracellular glucose levels result in a higher production of ROS within brain arterial endothelial cells (Figure 2). Also, my data reveals that changing extracellular glucose concentrations may cause a rapid increase in ROS production within BBECs. High glucose concentrations have been shown to result in an increase in PKC activity in human umbilical vein endothelial cells (HUVECs) which directly stimulates the production of ROS by cellular oxidases[8]. Also, mammary arteries[35] and cultured endothelial cells[54] have been shown to produce more ROS under high glucose. However, my data is unique as it investigated the effect of glucose on ROS production in brain arterial endothelial cells. The brain is a very important organ that demands a high amount of glucose to function correctly but too much glucose may be detrimental as it causes an increased production of ROS within the endothelium.

ROS sources in high glucose. The source of ROS production due to hyperglycemia may be a product of NADPH oxidase, uncoupled eNOS, the mitochondrial ETC, or XO. Typically, ROS production in endothelial cells is attributed to the NADPH oxidase, more specifically its NOX subunits[44,54,55]. The NOX subunit is believed to be the portion of the NADPH oxidase complex that generates ROS. NADPH is inhibited by DPI via reacting with NOX which prevents molecular oxygen from being converted to ROS. NADPH oxidase accounts for a relatively large amount of ROS produced due to glucose under static culture (Figure 5). However, NADPH oxidase contribution is severely reduced when high-glucose treated cultures are subjected to high flow. NADPH oxidase can be activated directly by PKC which itself can be activated under high glucose[56]. High glucose stimulates the activity of phospholipase C which cleaves phosphatidylinositol 4,5-bisphosphate into diacylglycerol[56]. Also, when glucose is in high concentrations, conversion to methylglyoxal can occur which is also a known activator of the NADPH complex[3]. High glucose, which has been shown to simulate hyperglycemic levels *in vivo*, has been shown to increase NADPH oxidase activity in bovine aortic endothelial cells (BAECs)[54], HUVECs[8], and animal models[57].

However, DPI is a non-specific inhibitor of flavoprotein function which can also be found in the mitochondrial electron transport chain[33,57,58]. Other flavoproteins that may be inhibited by DPI are eNOS, XO, and the SERCA pump which is responsible for calcium balance within myocytes[58]. However, no conclusive evidence has been presented that DPI significantly inhibits eNOS or XO. In my experiments, eNOS inhibition by DPI would have mirrored the effects of L-NAME on eNOS, which it did not. Likewise, XO inhibition by DPI would have mirrored the effects of febuxostat, which it did not. The SERCA pump is expressed in muscle cells, so inhibition of this protein would not have an effect on cultured endothelial cells.

However, I cannot discount that DPI inhibition may inhibit the mitochondrial ETC at Complex I[136,137].

Endothelial NOS was not found to contribute to the production of ROS due to high glucose. After eNOS inhibition using L-NAME, ROS levels were higher than in the untreated cultures of high and normal glucose (Figure 5). Therefore, eNOS was not a source of ROS production as inhibition of it would have caused a decrease in ROS. If eNOS were uncoupled and active, than inhibition using L-NAME would have resulted in the decrease of ROS due to the enzyme not functioning. Instead, ROS increased after eNOS inhibition which may speculate a possible function for eNOS in my system. Increased ROS levels may have resulted after eNOS inhibition because eNOS was contributing to the production of NO which provides bioavailable NO to the system.

In rats, hyperglycemia has been shown to uncouple eNOS and, in turn, increase the production of ROS[57]. Guzik et al found that eNOS inhibition in non-diabetic blood vessels resulted in the increased concentration of superoxide due to a decrease in NO bioavailability, but in diabetic blood vessels the release of superoxide was due to the generation of superoxide by uncoupled eNOS[35]. My findings are in contrast with those observations. The differences may arise between my experiment and the Guzik and Hink experiments because the model systems for experimentation were quite different. Guzik et al used blood vessels from human internal mammary arteries and human saphenous veins while Hink et al used rats induced with diabetes using streptozytocin while I used *in vitro* models of bovine brain arterial endothelial cells. The animal systems are very different from the *in vitro* method that I used. The differences between extracranial endothelial cells and intracranial endothelial cells is currently an intriguing field of study[25]. In human infants, intracranial endothelial cells showed increased resistance to

atherosclerosis than extracranial endothelial cells[28]. Atherosclerosis in brain arteries is seen in aging humans. Also, brain arterial endothelial cells contain more mitochondria that extracranial endothelial cells which may produce more ROS due to high glucose[25]. NADPH oxidase is also different between extracranial endothelial cells and arterial endothelial cells. Nox2 is highly expressed in extracranial endothelial cells whereas Nox4 is highly expressed in brain arterial endothelial cells[26,27].

Finally, it is important to note that cells in culture lose certain proteins and mechanisms[58]. For instance, endothelial cells in culture lose their von Willebrand Factor which is essential for cell adhesion and Factor VIII adhesion[59]. Endothelial cells *in vivo* also experience signaling from adjacent endothelial cells, signals from the extracellular matrix, chemicals in the blood, signaling from smooth muscle cells, and endocrine signaling which contribute to the physiology and function of the endothelial cell as a part of a system. Cultured endothelial cells only are in contact with the culture flask, growth medium, and adjacent cultured endothelial cells. Therefore, there are no extracellular signaling mechanisms that stimulate a process which contributes to the integrity of the blood vessel. For example, occlusion of one carotid artery will result in the expansion/hypertrophy of another which will restore near-normal blood flow to the brain[60].

When XO was inhibited in BBECs, ROS production was increased (Figure 5). XO inhibition has previously been shown to decrease ROS production within aortic endothelial cells[44] and rats[29]. Febuxostat is used to inhibit the function of XO, but not xanthine dehydrogenase, which is an inactive form of XO, which is very important to note[61]. Xanthine dehydrogenase can use the cofactor NADH to produce ROS and administration of febuxostat does not affect this generation[29,61]. This is quite intriguing because my data shows an increased production of ROS upon administration of febuxostat. It could be possible that XO was not

active during my experiment. Thus, inhibiting XO would have not done anything. If xanthine dehydrogenase was present, then this would explain the increased ROS production in BBECs.

The mitochondrial ETC is the main source of ROS production in high glucose (Figure 3 and 4). A very large percentage of ROS produced due to high glucose was attenuated with the administration of the complex IV inhibitor sodium azide. High glucose has been shown to increase mitochondrial superoxide formation in BAECs[38,62] and HUVECs[63]. Brian arterial endothelial cells have a greater amount of mitochondria than extracranial endothelial cells resulting in greater susceptibility to ROS generation under high glucose. Increased mitochondrial generation may be an initial cause to the various pathologies seen in brain arteries of diabetic patients. High glucose results in the increased activity of the mitochondrial ETC for energy production. While the metabolism of other macromolecules produces more energy per substrate, glucose metabolism happens quickly. Therefore, high levels of glucose should cause increased mitochondrial ROS production in BBECs.

ROS in response to flow. When endothelial cells sense flow, they are sensing mechanical stimulation in the form of wall shear stress (WSS). WSS is the frictional drag of blood exerted on the endothelium. The high flow that causes an increase in ROS production in BBECs (Figure 4) corresponds to a WSS of 10 Pa. High flow treatment of BBECs in high glucose caused a significant increase in ROS production compared to normal flow, but only increased ROS production slightly in normal glucose. However, normal flow did not cause an ROS production difference between high and normal glucose. Therefore, normal flow may be reducing the effect of glucose on ROS production. HUVECs have been shown to transiently increase ROS production when normal WSS is first applied (<1 hour), but ROS eventually returns to baseline levels (>1 hour)[29,64-66]. BAECs have also been shown to respond similarly to WSS[67]. High WSS

caused a high level of ROS production in both high and normal glucose which does not return to baseline ROS levels. High glucose is causing an exacerbated response by BBEC to high flow.

Normal flow in normal and high glucose resulted in very similar ROS levels (Figure 8). Normal WSS has been shown to increase the gene expression of protective proteins in endothelial cells. Topper et al found that normal flow upregulated the production of MnSOD which is located within the mitochondria[68]. MnSOD functions to convert mitochondrial superoxide to hydrogen peroxide which catalase can then convert to water decreasing the amount of ROS present. Similarly, Inoue et al showed that normal flow increased the expression of Cu/ZnSOD in HAECs[69]. Therefore, ROS concentration decreases under normal flow administration due to these proteins converting superoxide to hydrogen peroxide so that catalase can convert hydrogen peroxide to water. Chen et al investigated the response of antioxidant response element (ARE) gene regulation under normal flow and found that ARE genes expression in HAECs and HMECs[70]. Activation of ARE genes decreases oxidative stress within endothelial cells by neutralizing ROS. BBEC treated with normal flow might follow the same pattern of cellular protection preventing the elevation of superoxide in high glucose.

Another way to look at this is that high glucose exacerbates the response by BBEC to high WSS. There is a 3.5-fold increase in ROS production in BBEC treated with high glucose under high flow when compared to normal flow. High flow increased ROS 70% in normal glucose-treated BBECs under high WSS when compared to normal flow. Therefore, high glucose exacerbates the endothelial response to high WSS. Inhibition of NADPH oxidase resulted in a fairly large and significant inhibition of ROS in static BBEC. In normal glucose, inhibition of NADPH oxidase resulted in a 50% reduction of ROS production and ROS was decreased by 62% in high glucose (Figure 5). Therefore, NADPH oxidase significantly

contributed to ROS production in static culture. However, NADPH inhibition with DPI had nearly no effect when cultures were subjected to high flow (Figure 9). One explanation for NADPH oxidase not contributing to the WSS-induced ROS is that WSS could be inhibitory of NADPH function. WSS may inhibit NADPH oxidase function by preventing the regulatory unit $p47^{phox}$ from binding to the complex[71]. DeVerse et al showed that in human aortic endothelial cells under normal laminar WSS, ROS production by NADPH oxidase was minimal when compared to static culture[71].

It should be noted that DPI is not specific to the NADPH oxidase complex as it has been shown to slightly inhibit the mitochondrial ETC at complex I[33]. However, in my experiments, azide inhibition of the mitochondrial ETC had no effect in normal glucose (Figure 3 and 4). Therefore, the effect of DPI in normal glucose must not be due to an effect on the ETC.

There are a few theories pertaining to the possible mechanisms(s) of WSS detection. First, the platelet endothelial cell adhesion molecule-1 (PECAM-1) is a major shear stress sensor in endothelial cells. PECAM-1 is associated with adhesion between endothelial cells by a homophillic interaction with the PECAM-1 of an adjacent endothelial cell[72]. Integrin activation has long been believed to be a major sensory mechanism to mechanical stimuli such as WSS[13,15-17]. However, Tzima et al investigated an upstream complex of proteins that may be the initial detectors of WSS[14]. They found that PECAM-1 was probably the mechanosensor while VEGFR and VE-cadherin were important to the transduction of the signal[14]. VE-cadherin was believed to hold the complex together while VEGFR was believed to possibly act downstream[14].

When endothelial cells previously in a static environment experience WSS, cell-cell adhesions are stretched resulting in PECAM-1 being stretched which exposes phosphorylation

sites at two specific tyrosine residues (Tyr^{663} and Tyr^{686})[72]. Conway et al found that the administration of shear stress on BAECs increased the tension on PECAM-1 via FRET analysis[12]. PECAM-1 activation is thought to be an initiation step of signaling cascades in response to WSS. Fleming et al found that PECAM-1 was phosphorylated at Tyr^{663} and Tyr^{686} after being exposed to normal WSS[72]. This signal was transduced to eNOS and Akt via phosphorylation of Ser^{1177} and Ser^{473}, respectively[72]. Davies et al hypothesized that the cytoskeleton is important to the development of tension on PECAM-1 because of its direct connection and transmission properties with PECAM-1[13]. Essentially, they believed that the cytoskeleton was the primary sensory complex to WSS detection. However, Tzima et al found that the cytoskeleton was not the primary sensory mechanism to WSS in BAECs[73]. They found that cytoskeleton rearrangement occurred due to Rac1 activation which was a result of shear stress. Therefore, they concluded that the cytoskeleton does not sense mechanical stimulus further supporting the claim that PECAM-1 activation is an important protein which may sense mechanical stimuli.

Second, the glycocalyx of the endothelial cell may play an important role in the detection of WSS. The glycocalyx lies on the surface of the endothelium and consists of proteoglycans, glycoproteins, and absorbed plasma proteins. Heparan sulfate proteoglycans (HSPs) are believed to be the major component of the glycocalyx which respond to mechanical stimulation and a connected to the cytoskeleton[74-77]. Under normal WSS, the HSPs, particularly syndecans, penetrate into the cell and transmit mechanical stimuli to the cytoskeleton which activated results in the activation of eNOS via phosphorylation[74-78]. Enzymatic degradation of the HSPs abolished the shear-induced activation of eNOS[78]. Pahakis et al found that BAECs that have undergone degradation of the glycocalyx via enzymatic breakdown were not able to respond to flow by

eNOS activation. This suggests that the glycocalyx plays an important role in the detection and transduction of WSS[75,76]. Hyperglycemia decreases the volume of the glycocalyx making it more susceptible to high flow-induced ROS production by decreasing the activation of eNOS[78].

Third, stretch-activated ion channels are another hypothesized mechanism for WSS detection. Calcium may act as a second messenger to WSS detection via regulation of ion transport[79]. Injection of NaCl into cells caused an increased frequency of open ion channels increasing the intracellular concentrations of K^+ and Ca^{2+} [79]. Davies et al found that increased ion conductance was almost immediate upon subjection endothelial cells to WSS[80]. They proposed that WSS increases the pressure on cells which, due to the rigid cytoskeleton, causes ion channels to stretch and open resulting in an influx of ions from the external environment into the cytosol[80]. Normal WSS was shown to cause an influx of K^+ and Ca^{2+} into the cell milliseconds and seconds after administration of WSS to BAECs, HUVECs, HSVECs, and PAECs[80]. Increased ions, such as calcium, may increase the function of other proteins, such as NADPH oxidase which can be activated via Ca^{2+} [72]. Therefore, stretch-activated ion channels play a role in the detection of mechanical stimuli leading to the transduction and response intracellularly.

Implications in vascular disease. ROS in endothelial cells influence vascular damage and repair processes which play an important role in pathologies such as intracranial aneurysm (IA) formation and diabetic macrovascular complications[30,36,81]. First, ROS can damage biological molecules such as nucleic acids and proteins. Griendling et al have hypothesized that stable ROS (e.g. hydrogen peroxide) may also regulate gene expression levels by binding to nuclear receptors to increase gene expression[82]. Likewise, ROS modification of protein receptors may alter the function of the receptor[29]. ROS may also react with the DNA of cells causing activation of DNA repair mechanisms. Peroxynitrite is a potent initiator molecule of single-strand DNA

breaks and is created after the reaction of superoxide with NO[36,42]. Under DNA damage, poly(ADP) ribose polymerase (PARP) is activated and works to repair the single strand break[36,42]. Under PARP activation, cytokine and adhesion molecule production increases. In HUVECs, it was shown that PARP activation due to single-strand DNA breaks caused an increase in cytokines and adhesion molecules[42]. Cytokines stimulate the production of ROS intracellularly through PKC activation[32,41,83,84]. ROS, then, could stimulate the production of more ROS within endothelial cells via signaling or modification of proteins and/or DNA.

Second, ROS can stimulate inflammatory response molecules such as cytokines by means of the nuclear signaling molecule NF-κB[32,41,83,84]. ROS are known activators of NF-κB and can cause the κB portion to separate from the complex and translocate to the nucleus where it causes cytokine genes to be transcribed[83,84]. Further production of cytokines activate PKC which will increase intracellular ROS production through direct activation of intracellular oxidases such as NADPH oxidase[8], XO, and cyclooxygenase (mitochondrial)[32,83]. Some cytokines/inflammatory proteins, such as tumor necrosis factor-α (TNF-α), have been linked to the activation of MMPs due to hemodynamic flow[32].

Finally, ROS serve as a signaling molecule for processes such as MMP stimulation[32,34,82]. MMPs are Ca^{2+}- or Zn^{2+}-dependent endopeptidases that are secreted by many types of cells and, when activated, degrade extracellular matrix components[34]. This process is essential for the migration of endothelial cells during vascular remodeling and wound healing[86]. Hyperglycemia has also been shown to increase endothelial ROS production and MMP activation[34].

I have shown that treatment of BBEC with high glucose resulted in higher ROS production versus treatment with normal glucose. Diabetes Mellitus (DM) is a major risk factor

for many macrovascular complications in which an overwhelming amount of evidence suggests that there is involvement of ROS[8,34,36,81,87]. In diabetic rat models, Thum et al showed that eNOS was uncoupled in endothelial progenitor cells[43]. Uncoupling of eNOS switches from the typical function (producing NO) to the pathogenic function (producing ROS) as electrons are transferred to molecular oxygen instead of L-arginine[43]. ROS within endothelial cells may result in cellular dysfunction due to protein modifications, nucleic acid modifications, and inadvertent cellular signaling[30,81]. Endothelial dysfunction is believed to be one of the major precursor pathologies to macrovascular complications seen in diabetic patients[30,81]. As noted, ROS can stimulate the activity of MMPs which will degrade the ECM of endothelial cells as well as smooth muscle cells[32,34,82]. MMPs degrade the ECM which decreases the strength and resistance of the vascular wall allowing for an IA do further develop[32,34,82]. High glucose has been shown to increase the production of DAG, which will further activate PKC to increase MMP activity, in rats and BAECs[32,36]. As mentioned before, activation of PKC may, in turn, activate oxidases (NADPH, XO) to further produce ROS[38,41,83,84]. Hyperglycemia has been shown to increase PKC activity *in vivo*[2,34,56] and in BAECs[34].

I have also shown that high flow increases the production of ROS in BBEC. Increased ROS in brain arterial endothelial cells may result in the early pathogenesis of intracranial aneurysms (IA)[31]. In 2011, the Meng and Kolega lab found that treatment of rabbits with tempol, a radical scavenger, inhibited the induction of IA[31]. Meng and Kolega previously found that the endothelium of IA-induced rabbit models was intact and undamaged during the early stages of damage. The smooth muscle layer of the tunica media, however, was reduced in diameter. They also saw a thinning of the tunica media which created a weakened region of the blood vessel[31]. One possible explanation for this is that the ROS produced within the endothelial cell may be

exiting the endothelial layer and entering the tunica media where the ROS initiate apoptosis in smooth muscle cells. This could decrease the amount of smooth muscle cells in the tunica media causing the measured thinning of the tunica media. Vascular weakening allows IAs to form. Other laboratories have also shown that antioxidants can rescue the function of physiologically-impaired endothelial cells in diabetic blood vessels by restoring remodeling mechanisms previously impaired[81]. One explanation for this is that ROS stimulate the production of collagen type IV in the extracellular matrix which forms dense cross-links with one another[88,89]. Due to these cross-links, MMPs cannot degrade the matrix quickly resulting in a decreased ability for vascular remodeling to occur in DM patients[88,89]. The thinning of the tunica media and damage between the endothelial layer and the tunica media due to inflammation and matrix degradation could result in the weakening of the artery causing an aneurysm to form.

Future research may be garnered towards more mitochondrial specific research which would help us better understand the possible dysfunction of the mitochondria as well as its downstream effects within the brain arterial endothelial cell such as gene and protein expression. Glucose, in high concentrations, has been shown to non-enzymatically be added to cytosolic proteins which creates advanced glycation end-products[40]. Glucose could be bonded with mitochondrial membrane proteins resulting in a stimulation of ROS production, whether it be in the cytosol or the mitochondria. Advanced glycation end-products on the mitochondria could also change how the mitochondria functions. Since advanced glycation end-products result in increased ROS production in cells, it could be possible that the mitochondria will produce twice as much ROS as it is producing in my *in vitro* system.

Another protein that would be of interest would be cyclooxygenase which is complex IV of the mitochondrial ETC. It would be beneficial to know if increased ROS production is purely

due to increased activity of a normal concentration of protein or if it is due to an increased protein and/or gene expression of cyclooxygenase.

My research provides insight to the effects that glucose has on ROS production in endothelial cells. Understanding endothelial ROS production may allow future researchers to investigate how these may contribute to diabetic complications as they have not be researched extensively in DM models. ROS production may play a much larger role in the pathogenesis of DM complications than we currently believe, which is almost non-existent.

Works Cited

[1] Sheikh, Abdul Q., et al. "Angiogenic microenvironment augments impaired endothelial responses under diabetic conditions." American Journal of Physiology-Cell Physiology 306.8 (2014): C768-C778.

[2] Hink, Ulrich, et al. "Mechanisms underlying endothelial dysfunction in diabetes mellitus." Circulation research 88.2 (2001): e14-e22.

[3] Dikalov, Sergey. "Cross talk between mitochondria and NADPH oxidases." Free Radical Biology and Medicine 51.7 (2011): 1289-1301.

[4] Bourajjaj, M., et al. "Role of methylglyoxal adducts in the development of vascular complications in diabetes mellitus." Biochemical Society Transactions 31.6 (2003): 1400-1402.

[5] Su, Yang, et al. "Uncoupling of eNOS contributes to redox-sensitive leukocyte recruitment and microvascular leakage elicited by methylglyoxal." Biochemical pharmacology 86.12 (2013): 1762-1774.

[6] Miyazawa, Noriko, et al. "Methylglyoxal augments intracellular oxidative stress in human aortic endothelial cells." Free radical research 44.1 (2009): 101-107.

[7] Akhand, Anwarul A., et al. "Glyoxal and methylglyoxal trigger distinct signals for map family kinases and caspase activation in human endothelial cells." Free Radical Biology and Medicine 31.1 (2001): 20-30.

[8] Quagliaro, Lisa, et al. "Intermittent high glucose enhances apoptosis related to oxidative stress in human umbilical vein endothelial cells the role of protein kinase C and NAD (P) H-oxidase activation." Diabetes 52.11 (2003): 2795-2804.

[9] Li, Jian-Mei, and Ajay M. Shah. "ROS generation by nonphagocytic NADPH oxidase: potential relevance in diabetic nephropathy." Journal of the American Society of Nephrology 14.suppl 3 (2003): S221-S226.

[10] Liaw N, Dolan Fox JM, Siddiqui AH, Meng H, Kolega J (2014) Endothelial Nitric Oxide Synthase and Superoxide Mediate Hemodynamic Initiation of Intracranial Aneurysms. PLoS ONE 9(7): e101721. doi:10.1371/journal.pone.0101721

[11] Dolan, Jennifer M., John Kolega, and Hui Meng. “High Wall Shear Stress and Spatial Gradients in Vascular Pathology: A Review.” Annals of biomedical engineering 41.7 (2013): 1411–1427. PMC. Web. 13 Mar. 2015.

[12] Conway, Daniel E., et al. "Fluid shear stress on endothelial cells modulates mechanical tension across VE-cadherin and PECAM-1." *Current Biology*23.11 (2013): 1024-1030.

[13] Davies, Peter F., and Kenneth A. Barbee. "Endothelial cell surface imaging: insights into hemodynamic force transduction." *Physiology* 9.4 (1994): 153-157.

[14] Tzima, Eleni, et al. "A mechanosensory complex that mediates the endothelial cell response to fluid shear stress." *Nature* 437.7057 (2005): 426-431.

[15] Burridge, Keith, and Magdalena Chrzanowska-Wodnicka. "Focal adhesions, contractility, and signaling." *Annual review of cell and developmental biology* 12.1 (1996): 463-519.

[16] Dejana, Elisabetta. "Endothelial cell adhesive receptors." *Journal of cardiovascular pharmacology* 21 (1993): S18-S21.

[17] Takahashi, Masafumi, and Bradford C. Berk. "Mitogen-activated protein kinase (ERK1/2) activation by shear stress and adhesion in endothelial cells. Essential role for a herbimycin-sensitive kinase." *Journal of Clinical Investigation* 98.11 (1996): 2623.

[18] Wiebers, David O., and International Study of Unruptured Intracranial Aneurysms Investigators. "Unruptured intracranial aneurysms: natural history, clinical outcome, and risks of surgical and endovascular treatment." The Lancet 362.9378 (2003): 103-110.

[19] "NINDS Cerebral Aneurysms Information Page." Cerebral Aneurysms Information Page: National Institute of Neurological Disorders and Stroke (NINDS). Web. 11 Feb. 2015. <http://www.ninds.nih.gov/disorders/cerebral_aneurysm/cerebral_aneurysms.htm>.

[20] Jaclyn M. Alfano, John Kolega, Sabareesh K. Natarajan, Jianping Xiang, Rocco A. Paluch,Elad I. Levy, Adnan H. Siddiqui, Hui Meng. (2013) Intracranial Aneurysms Occur More Frequently at Bifurcation Sites That Typically Experience Higher Hemodynamic Stresses.*Neurosurgery* **73**, 497-505. . Online publication date: 1-Sep-2013.

[21] Khurana, Vini, and Robert Spetzler. *The Brain Anuerysm: A Comprehensive Resource for Brain Aneurysm Patients, Their Families, and Physicians*. Phoenix: AuthorHouse, 2006. Print.

[22] Mathews, Christopher K. Biochemistry. 4th ed. Toronto: Pearson, 2013. Print.

[23] Gartner, Leslie P., and James L. Hiatt. *Color Textbook of Histology*. 3rd ed. Philadelphia, PA: Saunders/Elsevier, 2007. Print.

[24] Young, Barbara. *Wheater's Functional Histology: A Text and Colour Atlas*. 5th ed. Edinburgh: Churchill Livingstone/Elsevier, 2006. Print.

[25] Rizzo, Maria Teresa, and H. Anne Leaver. "Brain endothelial cell death: modes, signaling pathways, and relevance to neural development, homeostasis, and disease." Molecular neurobiology 42.1 (2010): 52-63.

[26] Ago, Tetsuro, et al. "NAD (P) H oxidases in rat basilar arterial endothelial cells." Stroke 36.5 (2005): 1040-1046.

[27] Miller, Alyson A., et al. "NADPH oxidase activity and function are profoundly greater in cerebral versus systemic arteries." Circulation research 97.10 (2005): 1055-1062.

[28] Napoli, Claudio, et al. "Intracranial arteries of human fetuses are more resistant to hypercholesterolemia-induced fatty streak formation than extracranial arteries." *Circulation* 99.15 (1999): 2003-2010.

[29] Cai, Hua, and David G. Harrison. "Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress." *Circulation research* 87.10 (2000): 840-844.

[30] Deanfield, John E., Julian P. Halcox, and Ton J. Rabelink. "Endothelial function and dysfunction testing and clinical relevance." Circulation 115.10 (2007): 1285-1295.

[31] Meng, Hui, et al. "Progressive aneurysm development following hemodynamic insult: laboratory investigation." *Journal of neurosurgery*114.4 (2011): 1095-1103.

[32] Chalouhi, Nohra, et al. "Biology of intracranial aneurysms: role of inflammation." Journal of Cerebral Blood Flow & Metabolism 32.9 (2012): 1659-1676.

[33] Schaffer, Stephen W., and M. Saadeh Suleiman. Mitochondria: the dynamic organelle. Vol. 2. Springer Science & Business Media, 2010.

[34] Uemura, Shiro, et al. "Diabetes mellitus enhances vascular matrix metalloproteinase activity role of oxidative stress." Circulation Research 88.12 (2001): 1291-1298.

[35] Guzik, Tomasz J., et al. "Mechanisms of increased vascular superoxide production in human diabetes mellitus Role of NAD (P) H oxidase and endothelial nitric oxide synthase." Circulation 105.14 (2002): 1656-1662.

[36] Brownlee, Michael. "The pathobiology of diabetic complications a unifying mechanism." Diabetes 54.6 (2005): 1615-1625.

[37] Newsholme, P., et al. "Diabetes associated cell stress and dysfunction: role of mitochondrial and non-mitochondrial ROS production and activity." The Journal of physiology 583.1 (2007): 9-24.

[38] Nishikawa, Takeshi, et al. "Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage." Nature 404.6779 (2000): 787-790.

[39] Bugger, Heiko, and E. Dale Abel. "Mitochondria in the diabetic heart." Cardiovascular research (2010): cvq239.

[40] Rosca, Mariana G., et al. "Glycation of mitochondrial proteins from diabetic rat kidney is associated with excess superoxide formation." American Journal of Physiology-Renal Physiology 289.2 (2005): F420-F430.

[41] Taniyama, Yoshihiro, and Kathy K. Griendling. "Reactive oxygen species in the vasculature molecular and cellular mechanisms." *Hypertension* 42.6 (2003): 1075-1081.

[42] Piconi L, Quagliaro L, Da Ros R, Assaloni R, Giugliano D, Esposito K, Szabo´ C, Ceriello A. Intermittent high glucose enhancesICAM-1, VCAM-1, E-selectin and interleukin-6 expression in human umbilical endothelial cells in culture: the role of poly(ADP-ribose) polymerase.J Thromb Haemost 2004; 2: 1453–9.

[43] Thum, Thomas, et al. "Endothelial nitric oxide synthase uncoupling impairs endothelial progenitor cell mobilization and function in diabetes." Diabetes 56.3 (2007): 666-674.

[44] McNally, J. Scott, et al. "Role of xanthine oxidoreductase and NAD (P) H oxidase in endothelial superoxide production in response to oscillatory shear stress." American Journal of Physiology-Heart and Circulatory Physiology 285.6 (2003): H2290-H2297.

[45] Alnæs, Martin Sandve, et al. "Computation of hemodynamics in the circle of Willis." *Stroke* 38.9 (2007): 2500-2505.

[46] Lindekleiv, Haakon M., et al. "Sex differences in intracranial arterial bifurcations." *Gender medicine* 7.2 (2010): 149-155.

[47] Traub, Oren, and Bradford C. Berk. "Laminar shear stress mechanisms by which endothelial cells transduce an atheroprotective force."*Arteriosclerosis, thrombosis, and vascular biology* 18.5 (1998): 677-685.

[48] Selective fluorescent imaging of superoxide in vivo using ethidium-based probes. Robinson KM, Janes MS, Pehar M, Monette JS, Ross MF, Hagen TM, Murphy MP, Beckman JS Proc Natl Acad Sci U S A (2006) 103:15038-15043.

[49] The molecular probes handbook: a guide to fluorescent probes and labeling technologies. Technologies Corporation, 2010.

[50] Abramoff, M.D., Magalhaes, P.J., Ram, S.J. "Image Processing with ImageJ". Biophotonics International, volume 11, issue 7, pp. 36-42, 2004.

[51] "A Closer Look at Mitochondrial Dysfunction With MitoSOX™ Red Mitochondrial Superoxide Indicator." *A Closer Look at Mitochondrial Dysfunction With MitoSOX™ Red Mitochondrial Superoxide Indicator*. Web. 27 Apr. 2015. <http://www.lifetechnologies.com/us/en/home/references/newsletters-and-journals/bioprobes-journal-of-cell-biology-applications/bioprobes-issues-2010/bioprobes-62/mitochondrial-dysfunction-and-mitosox-red-mitochondrial-superoxide-indicator.html>.

[52] Schwoebel, Eric D., Thai H. Ho, and Mary Shannon Moore. "The Mechanism of Inhibition of Ran-Dependent Nuclear Transport by Cellular ATP Depletion." The Journal of Cell Biology 157.6 (2002): 963–974. PMC. Web. 23 Mar. 2015.

[53] Malek, A. M., S. L. Alper, and S. Izumo. Hemodynamic shear stress and its role in atherosclerosis. JAMA 282:2035–2042, 1999.

[54] Inoguchi, Toyoshi, et al. "High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C--dependent activation of NAD (P) H oxidase in cultured vascular cells." *Diabetes* 49.11 (2000): 1939-1945.

[55] ur Rehman, Atif, et al. "Selective inhibition of NADPH oxidase reverses the over contraction of diabetic rat aorta." *Redox biology* 2 (2014): 61-64.

[56] Gutterman, David D. "Vascular Dysfunction in Hyperglycemia Is Protein Kinase C the Culprit?." *Circulation research* 90.1 (2002): 5-7.

[57] Hink, Ulrich, et al. "Mechanisms underlying endothelial dysfunction in diabetes mellitus." Circulation research 88.2 (2001): e14-e22.

[58] Li, Yunbo, and Michael A. Trush. "Diphenyleneiodonium, an NAD (P) H oxidase inhibitor, also potently inhibits mitochondrial reactive oxygen species production." *Biochemical and biophysical research communications* 253.2 (1998): 295-299.

[59] Sadler, J. Evan. "Biochemistry and genetics of von Willebrand factor."*Annual review of biochemistry* 67.1 (1998): 395-424.

[60] Kuroda, Satoshi, et al. "Long-Term Prognosis of Medically Treated Patients With Internal Carotid or Middle Cerebral Artery Occlusion Can Acetazolamide Test Predict It?." *Stroke* 32.9 (2001): 2110-2116.

[61] Sanders, Stephen A., Robert Eisenthal, and Roger Harrison. "NADH oxidase activity of human xanthine oxidoreductase." *European Journal of Biochemistry* 245.3 (1997): 541-548.

[62] Giugliano, Dario, Antonio Ceriello, and Giuseppe Paolisso. "Diabetes mellitus, hypertension, and cardiovascular disease: which role for oxidative stress?." Metabolism 44.3 (1995): 363-368.

[63] Kukidome, Daisuke, et al. "Activation of AMP-activated protein kinase reduces hyperglycemia-induced mitochondrial reactive oxygen species production and promotes mitochondrial biogenesis in human umbilical vein endothelial cells." *Diabetes* 55.1 (2006): 120-127.

[64] De Keulenaer, Gilles W., et al. "Oscillatory and steady laminar shear stress differentially affect human endothelial redox state role of a superoxide-producing NADH oxidase." Circulation research 82.10 (1998): 1094-1101.

[65] Chiu, J. J., et al. "Reactive oxygen species are involved in shear stress-induced intercellular adhesion molecule-1 expression in endothelial cells." *Arteriosclerosis, Thrombosis, and Vascular Biology* 17.12 (1997): 3570-3577.

[66] Hsieh, Hsyue-Jen, et al. "Increase of reactive oxygen species (ROS) in endothelial cells by shear flow and involvement of ROS in shear-induced c-fos expression." *Journal of cellular physiology* 175.2 (1998): 156-162.

[67] Dolan, Jennifer M., et al. "Endothelial cells express a unique transcriptional profile under very high wall shear stress known to induce expansive arterial remodeling." *American Journal of Physiology-Cell Physiology* 302.8 (2012): C1109-C1118.

[68] Topper, James N., et al. "Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress."*Proceedings of the National Academy of Sciences* 93.19 (1996): 10417-10422.

[69] Inoue, Nobutaka, et al. "Shear stress modulates expression of Cu/Zn superoxide dismutase in human aortic endothelial cells." *Circulation research* 79.1 (1996): 32-37.

[70] Chen, Xi-Lin, et al. "Laminar Flow Induction of Antioxidant Response Element-mediated Genes in Endothelial Cells A NOVEL ANTI-INFLAMMATORY MECHANISM." Journal of Biological Chemistry 278.2 (2003): 703-711.

[71] DeVerse, J. Sherrod, et al. "Shear stress modulates RAGE-mediated inflammation in a model of diabetes-induced metabolic stress." American Journal of Physiology-Heart and Circulatory Physiology 302.12 (2012): H2498-H2508.

[72] Fleming, Ingrid, et al. "Role of PECAM-1 in the shear-stress-induced activation of Akt and the endothelial nitric oxide synthase (eNOS) in endothelial cells." *Journal of cell science* 118.18 (2005): 4103-4111.

[73] Tzima, Eleni, et al. "Activation of integrins in endothelial cells by fluid shear stress mediates Rho-dependent cytoskeletal alignment." *The EMBO journal*20.17 (2001): 4639-4647.

[74] Brower, Jeremy Benjamin. High Glucose Impairs the Endothelial Shear Stress Response. Order No. 3361834 Arizona State University, 2009 Ann ArborProQuest. 26 Mar. 2015.

[75] Pahakis, Manolis Y., et al. "The role of endothelial glycocalyx components in mechanotransduction of fluid shear stress." *Biochemical and biophysical research communications* 355.1 (2007): 228-233.

[76] Tarbell, John M., and M. Y. Pahakis. "Mechanotransduction and the glycocalyx." *Journal of internal medicine* 259.4 (2006): 339-350.

[77] Jacob, Matthias, et al. "The endothelial glycocalyx prefers albumin for evoking shear stress-induced, nitric oxide-mediated coronary dilatation."*Journal of vascular research* 44.6 (2007): 435-443.

[78] Nieuwdorp, Max, et al. "Loss of endothelial glycocalyx during acute hyperglycemia coincides with endothelial dysfunction and coagulation activation in vivo." *Diabetes* 55.2 (2006): 480-486.

[79] Sackin, Henry. "Stretch-activated ion channels." *Kidney international* 48.4 (1995): 1134-1147.

[80] Davies, Peter F., and S. C. Tripathi. "Mechanical stress mechanisms and the cell. An endothelial paradigm." *Circulation research* 72.2 (1993): 239-245.

[81] Spinetti, Gaia, et al. "Diabetes and vessel wall remodeling: from mechanistic insights to regenerative therapies." Cardiovascular research (2008).

[82] Griendling, Kathy K., and Garret A. FitzGerald. "Oxidative stress and cardiovascular injury part I: basic mechanisms and in vivo monitoring of ROS." *Circulation* 108.16 (2003): 1912-1916.

[83] Olmez, Inan, and Huseyin Ozyurt. "Reactive oxygen species and ischemic cerebrovascular disease." Neurochemistry international 60.2 (2012): 208-212.

[84] Lawrence, Toby. "The nuclear factor NF-κB pathway in inflammation." *Cold Spring Harbor perspectives in biology* 1.6 (2009): a001651.

[85] Yang, Dongli, et al. "Pro-inflammatory cytokines increase reactive oxygen species through mitochondria and NADPH oxidase in cultured RPE cells."*Experimental eye research* 85.4 (2007): 462-472.

[86] Huang, Hayden, Roger D. Kamm, and Richard T. Lee. "Cell mechanics and mechanotransduction: pathways, probes, and physiology." *American Journal of Physiology-Cell Physiology* 287.1 (2004): C1-C11.

[87] Waeber, François Feihl, Luis Ruilope, Bernard. "Diabetes and hypertension."*Blood pressure* 10.5-6 (2001): 311-321.

[88] Shantikumar, S., et al. "Diabetes and the abdominal aortic aneurysm." European Journal of Vascular and Endovascular Surgery 39.2 (2010): 200-207.

[89] Cagliero, Enrico, et al. "Increased expression of basement membrane components in human endothelial cells cultured in high glucose." *Journal of Clinical Investigation* 82.2 (1988): 735.

[90] Ellis, Juliet A., Susan J. Mayer, and O. T. Jones. "The effect of the NADPH oxidase inhibitor diphenyleneiodonium on aerobic and anaerobic microbicidal activities of human neutrophils." *Biochem. J* 251 (1988): 887-891.

[91] Florian, Jeffry A., et al. "Heparan sulfate proteoglycan is a mechanosensor on endothelial cells." *Circulation research* 93.10 (2003): e136-e142.