Mechanisms of Hyperglycemia-Induced ROS Production in Osmotically Swollen Glial Cells

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MECHANISMS OF HYPERGLYCEMIA-INDUCED ROS PRODUCTION IN OSMOTICALLY SWOLLEN GLIAL CELLS

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

By

AUGUSTA KWANSIMA EDUAFO
B.S., University of Dayton, 2011

2015
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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Augusta Kwansima Eduafu ENTITLED Mechanisms Of Hyperglycemia-Induced Ros Production In Osmotically Swollen Glial Cells BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF Master Of Science.

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ABSTRACT


Cerebral edema is a potentially fatal complication of diabetes. There is evidence for increased Reactive Oxidative Species (ROS) production during cerebral edema in diabetics. This can lead to oxidative stress, thought to contribute to the onset and progression of diabetes and can cause cell injury and cell death. ROS, in moderate amounts, are involved in physiological processes within the body that produce favorable cellular responses. Unfortunately, high levels of ROS can lead to cellular damage of lipids, membranes, proteins and DNA. Hyperglycemia can cause an increase in ROS production due to activation of NADPH oxidases. The purpose of this study is to determine if there is increased ROS production in swollen hyperglycemic cells thus leading to cell injury and also to determine the mechanism by which this increased ROS production occurs. C6 rat glioma cells were grown in normal glucose medium or were exposed to high glucose medium for 24 hours to simulate diabetic conditions. Cells were then perfused with isoosmotic and hypoosmotic PBS and ROS production was measured over a 30-minute experiment. There was no change in ROS production when cells grown in normal glucose (normoglycemic) medium were osmotically swollen. Cells grown in high glucose (hyperglycemic) medium had increased basal ROS production compared with cells grown in normal glucose medium. These cells further increased their ROS production when osmotically swollen. Blocking Nox2 and Nox4 enzyme activity decreased basal ROS production of cells grown either in normoglycemic or hyperglycemic conditions. Blocking Nox2 and Nox4 also decreased ROS production of
hyperglycemic cells during hypoosmotic exposure. However, cells grown in normal glucose medium increased their production of ROS when osmotically swollen in the presence of Nox2 and Nox4 inhibitors. Western blot analysis was also done to determine expression of eNos in hyperglycemic cells as compared to normoglycemic cells. There was no difference in expression of eNos in hyperglycemic cells compared to normoglycemic cells.

Normoglycemic cells did not increase their ROS production in hypoosmotic conditions. Hyperglycemic cells had increased ROS production in isoosmotic conditions. This increase in ROS production is even greater when hyperglycemic cells are swollen. The increase in ROS production in hyperglycemic cells may be due to Nox, but it’s not due to eNos. Swelling hyperglycemic cells in normoglycemic conditions causes mild cell injury. Hyperglycemia, a major risk factor of diabetes, causes cellular injury but even more so when there is cellular edema.

This work appeared in abstract form at the Ohio Miami Valley Chapter of the Society for Neuroscience meeting 2015.
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LIST OF ABBREVIATIONS

1. ROS - Reactive Oxidative Species
2. DKA - Diabetic Ketoacidosis
3. C6 Cells - Rat C6 Glioma Cells
4. RVI - Regulatory Volume Increase
5. RVD - Regulatory Volume Decrease
6. Nox2 - NADPH Oxidase 2
7. Nox 4 - NADPH Oxidase 4
8. DCF - 2',7'-dichlorofluorescein
9. DHE - Dihydroethidium
10. DPI - Diphenyleneiodonium
11. eNos - Endothelial Nitric Oxide Synthase
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INTRODUCTION
**Pathophysiology**

Diabetes is a complex disorder characterized by defects in the body’s ability to control glucose and insulin homeostasis and has become a major public health issue (37). Over 29 million Americans (9.3% of the U.S. population) were estimated to have diabetes in 2012 (37). This is a 1.5% increase from data accumulated in 2007. The estimated total economic cost of diagnosed diabetes in 2012 was $245 billion. It imposes a substantial burden on the economy of the U.S in the form of increased medical costs and indirect costs from work-related absenteeism, reduced productivity at work and at home, reduced labor participation from chronic disability, and premature mortality (53). It is known that diabetes has harmful effects on the nervous system. This can include peripheral and autonomic neuropathy and cranial neuropathy, affecting nerves that control the extraocular muscles of the eye. Persistent hyperglycemia seems to play an important role in cerebral dysfunction affecting memory function and cognitive reasoning (28, 42). Chronic hyperglycemia also may reduce white matter and gray matter volume (42).

There are two main types of diabetes. Type I diabetes occurs when the immune system attacks and destroys insulin-producing beta cells in the pancreas. Without insulin glucose becomes elevated in the bloodstream. Type I diabetes is commonly called insulin-dependent because those affected can receive insulin injections to maintain normal blood glucose concentrations. Type II diabetes occurs when cells do not use the insulin properly thus causing the body to develop an insulin resistance. Type II diabetes accounts for more than 90% of individuals diagnosed with diabetes (37). Like Type I diabetes, Type II diabetes causes
increased glucose concentrations in the blood. Though there are different causes for these types of diabetes a common result of both types of diabetes is hyperglycemia.

DKA

Diabetic Ketoacidosis (DKA) is a rare yet potentially fatal hyperglycemic crisis that can occur in patients with either Type I or Type II diabetes. Insulin resistance and insulin deficiency along with hyperglycemia, ketosis, dehydration, and electrolyte imbalance underlie the pathophysiology of DKA (17, 18). Reports have also shown that high levels of ketone bodies can increase cellular oxidative stress, which may contribute to the development of insulin resistance seen in both types of diabetes (37). Brain edema is a potentially fatal complication of DKA. There is much controversy over the rehydration methods that will cause the least complications when treating DKA. At the center of the controversy surrounding DKA treatment are physicians’ concerns about possibly causing or exacerbating DKA-related cerebral edema or cerebral injury with inappropriate IV rehydration (17,18,49). There is still little known about the exact mechanism of cerebral edema but it can be associated with the disruption of tight junction proteins of the blood brain barrier (22). Clinically overt cerebral edema occurs in 0.5-1% of DKA episodes and has a high mortality rate of 21-24%. Survivors frequently are left with permanent neurological deficits (17, 18, 49).

Brain Edema

Brain edema is characterized as an abnormal accumulation of fluid within the brain parenchyma, producing a volumetric enlargement of the brain cells or tissue (24). There are two types of brain edema, vasogenic and cellular edema. Vasogenic
edema occurs when there is injury to the blood vessel walls causing increased permeability of the blood brain barrier. Cellular edema occurs when there is intracellular swelling without increased permeability of the blood brain barrier. My research focuses primarily on cellular edema. The cells most readily affected by cellular edema are astrocytes.

Astroglial swelling has been studied during ischemia and trauma in considerable detail. Swelling of astroglial cells is a very complex phenomenon and may occur by various mechanisms. For example, swelling can result from simultaneous operation of Cl⁻/HCO₃⁻ and Na⁺/H⁺ exchange transporters. If the increased intracellular NaCl is not pumped out by some other transport process, water will flow into the cell (26). Glutamate can also cause swelling of astroglial cells. Astroglia taken up glutamate is by a Na⁺ and K⁺ dependent mechanism. There has also been evidence of the role of free radicals as cell swelling mediators in astroglial and other cells. Free radicals such as superoxide lead to lipid peroxidation and breakdown of phospholipids, which in turn leads to the breakdown of the cell plasma membrane. The breakdown of the plasma membrane could cause an influx of Na and anions, causing swelling (26).

Multiple mechanisms may cause cellular edema with resulting numerous clinical impacts on the brain. Edema can affect cerebral arterial perfusion causing ischemia. In extreme situations, this may lead to brain herniation once intracranial pressure reaches a critical level (24, 26). My research will provide more knowledge about the deleterious effects of cellular edema.
**Cell Volume Regulation**

Cell volume regulation is an important aspect of cell physiology. Cells must be able to regulate their volume in isoosmotic conditions as well as anisosmotic conditions. The mammalian cell membrane, with a few exceptions, is highly permeable to water. When too much water moves into the cell due to a changing intracellular or extracellular osmotic environment, the cell swells and potentially can rupture. If too much water leaves the cell, then the internal environment will become too concentrated with ions and the cell will not be able to carry out its normal functions. Cells control their volume by changing the intracellular osmolyte content through the movement of ions and organic osmolytes across the plasma membrane. For example, osmotically swollen cells release K⁺, Cl⁻, nonessential organic osmolytes, and cell water, thereby reducing the cell volume towards the original value, the process termed regulatory volume decrease (RVD). Osmotically shrunken cells generally initiate a net gain of KCl and organic osmolytes with resulting influx of water, thereby increasing cell volume towards the original value, the process termed regulatory volume increase (RVI) (21).

In steady state conditions, there is constant risk to cell volume equilibrium due to entrance of diffusible ions and water. Cells have mechanisms in place that counteract these volume perturbations. The Gibbs-Donnan equilibrium states that the sum of the monovalent cations times sum of the monovalent anions on one side of the membrane is equal to the products of the activities of the sum of these ions on the other side of the membrane. Thus at equilibrium, with electroneutrality
maintained on both sides of the membrane, and for a simple solution containing only intracellular (i) and extracellular (o) sodium, potassium and chloride:

\[
([\text{Na}^+] + [\text{K}^+])_i \times [\text{Cl}^-]_i = ([\text{Na}^+] + [\text{K}^+])_o \times [\text{Cl}^-]_o
\] (10)

However in mammalian cells, an osmotic pressure is unavoidably developed across the cell membrane due to impermeable charged intracellular macromolecules. Consequently hydrostatic pressure would be needed to balance the osmotic pressure. Mammalian cell volume homeostasis can be described by a system similar to the Gibbs-Donnan equilibrium and known as the double Donnan equilibrium. With the double Donnan system, an extracellular solute, such as sodium, can balance the osmotic pressure of the intracellular macromolecules. In order for this system to work, the cell must be effectively impermeable to this solute. Low plasma membrane sodium permeability and the Na\(^+/\)K\(^+\) ATPase generate a gradient of sodium concentration across the plasma membrane. Sodium is pumped out of the cell, against its gradient, and potassium is pumped into the cell via the Na\(^+/\)K\(^+\) ATPase. Outward transport of sodium maintains a low intracellular concentration and high extracellular concentration of sodium. (10)

Disruption of the double Donnan equilibrium due to anisoosmotic conditions can cause the cell to swell or shrink as it regains osmotic balance across the plasma membrane. Once most mammalian cells are exposed to a change in osmolarity they activate processes to return the cell to normal size. These processes result in RVI when cells are osmotically shrunken and RVD when cells are osmotically swollen.

*Regulatory Volume Increase*
Two known mechanisms that regulate volume after cell shrinkage are the Na\(^+\)/H\(^+\) exchanger combined with Cl\(^-\)/HCO\(_3\)\(^-\) exchanger and the Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter (10). Na\(^+\)/H\(^+\) exchangers (NHE) are found in essentially all organisms. NHEs are complexly regulated, multifunctional proteins mediating the one to one exchange of Na\(^+\) for H\(^+\) across membranes. The mechanism of NHE activation during cell shrinkage is incompletely understood, though a number of signaling pathways have been implicated (10). In the presence of HCO\(_3\)\(^-\), NHE contribute significantly to RVI. When HCO\(_3\) is absent, the Na\(^+\)-K\(^+\)-2Cl\(^-\) co-transporter is the primary process for RVI. This mechanism first was seen in Ehrlich ascites tumor (EAT) cells (10).

Regulatory Volume Decrease

The solute most commonly lost in osmotically swollen mammalian cells is KCl. In regulatory volume decrease swelling-activated Cl\(^-\) and K\(^+\) channels facilitate solute loss. The coordinated action of K\(^+\) and Cl\(^-\) channels brings about the efflux of KCl and the consequent water efflux due to intracellular reduction of osmolytes (41). In EAT cells Cl\(^-\) flux increased dramatically during swelling and was paralleled with a K\(^+\) flux resulting in a net KCl efflux. In addition to release of inorganic solutes, most cells also release organic solutes from the cell during the RVD process. For example, primary astrocytes have been shown to release glutamate, aspartate, and taurine following hypotonic swelling (23). In C6 rat glioma cells, the RVD response involves K\(^+\) efflux from the cell and a loss of KCl. Though the mechanism has not been clearly defined, stretch activated channels, the cytoskeleton, and intracellular signaling pathways also may play important roles in RVD (21).
Oxidative Species

Oxidative species have been seen as potential contributors to both the onset and the progression of diabetes and its associated complications (37). Reactive Oxygen Species (ROS) are formed from the reduction of molecular oxygen or by the oxidation of water to yield products such as superoxide, hydrogen peroxide and hydroxyl radical. Oxidative stress occurs when production of these free radicals exceeds the cellular antioxidant capacity. If not removed, ROS will attack proteins, lipids and nucleic acids. In a biological system, the mitochondria and NADPH oxidase are the major sources of ROS production (37).

Reactive Oxidative Species

Reactive Oxygen Species are made within cells and are essential for normal physiology, but also are believed to accelerate aging and to mediate cellular degeneration in disease states (47). ROS are important for maintaining vascular diameter and vascular cell function, mounting effective immune responses, acting as possible signaling molecules in regulating skeletal muscle glucose uptake, and regulating gene stability and transcription by affecting chromatin stability (2).

The mitochondrial electron transport chain represents the major source of cellular ROS production. Almost all mammalian cells and tissues continuously convert a small proportion of molecular oxygen into superoxide anion by the univalent reduction of molecular oxygen in the electron transport chain (2). The superoxide anion is nonenzymatically formed by the electron transport chain semiubiquinone compound and then enzymatically converted to hydrogen peroxide by superoxide dismutase (SOD). ROS generation in the mitochondria is under tight
control by various mechanisms among which are the uncoupling proteins. By diminishing the formation of a large proton gradient, these proteins located in the inner mitochondrial matrix are considered natural regulators of ROS production in the mitochondria (2).

**ROS production in hyperglycemia**

Hyperglycemia, or high blood glucose, is a major concern for diabetics. Frequent or ongoing high blood glucose can cause damage to nerves, blood vessels, and organs. Hyperglycemia also has adverse effects on the brain. Research with astrocytes has shown that hyperglycemia induces a significant increase in ROS production concurrent with the activation of cytokines, specifically NF-κB and STAT 3 (45). These cytokines are activated in a ROS dependent manner. In astrocytes exposed to high glucose, NF-κB and STAT 3, which are generally localized in the cytoplasm, are translocated in the nucleus. Inhibition of ROS production in the astrocytes reduces the high glucose-induced cytokine expression in the nucleus (48).

A large amount of data emphasizes six key metabolic pathways as being major contributors to hyperglycemia-induced cell damage; sorbitol metabolism, hexosamine metabolism, dicarbonyl formation and glycation enolization and α-ketoaldehyde formation, oxidative phosphorylation, and protein kinase C activation (53). Protein kinase C (PKC) activation has been shown to be a potent activator of NADPH oxidase, which is a known mechanism of ROS production (37). My research will further examine the mechanisms by which ROS is produced in hyperglycemic cells.
**NADPH (Polyol) pathway**

The NADPH pathway has been shown to be a major contributor to ROS production. The pathway leads to reduction of glucose to sorbitol via aldose reductase in an NADPH dependent manner. Aldose reductase also reduces toxic aldehydes formed by ROS or other substrates to inactive alcohols. Under hyperglycemic conditions, aldose reductase has a greater affinity for glucose. The over production of sorbitol in hyperglycemic conditions results in an overall decrease in NADPH, an essential cofactor for the production of glutathione, a critical intracellular antioxidant. Thus while increased glucose through the polyol pathway does not produce ROS directly, it can contribute greatly to an overall redox imbalance in the cell that leads to oxidative stress (37).

**NADPH Oxidase Complex**

Superoxide radicals can be produced by reactions catalyzed by xanthine oxidase, NADPH oxidase and cytochrome P450. For the purpose of this research the main focus will the NADPH oxidase complexes. The NADPH oxidase is an electron transporter that catalyzes the NADPH-dependent reduction of oxygen to the superoxide anion (\(O_2^-\)) (6, 7, 11, 27). This ROS-generating enzyme was first found in phagocytes (macrophages, monocytes and neutrophils) as a first line of defense against bacteria and other potential threats and was named phagocyte oxidase (6, 7, 11, 27). Later research showed that nonphagocytic cells could also produce \(O_2^-\) and \(H_2O_2\). Data suggested that the cytochrome in nonphagocytic cells is genetically distinct from the phagocyte flavocytochrome b558 (6, 7). Eventually more research identified seven homologs of the phagocyte NADPH oxidase in mammals, which
were identified as the Nox/Duox family, consisting of Nox1, Nox2, Nox3, Nox4, Nox5, Duox1 and Duox2. These oxidases share a common core structure but differ in their functions, regulatory mechanisms, tissues and subcellular patterns of expression, and downstream targets (6, 7, 11, 27). The NADPH oxidases are found in neurons, endothelial cells, vascular smooth muscle cells, macrophages, adventitial fibroblasts, cardiac myocytes and fibroblasts, as well as adipocytes and stem cells (6).

The control of Nox enzyme activity is exerted by calcium or protein-protein interactions and concerns all Nox enzymes with the exception of Nox4, which is constitutively active (7). Nox5 and the Duox enzymes are directly calcium dependent while Nox1 to Nox3 are not directly calcium dependent although calcium is involved in the upstream mechanism of their activation (7). Two other elements contribute to the activation of Nox enzyme activity; a small GTPase Rac and cytosolic activator proteins. Under resting conditions the phagocyte oxidase, consisting of the integral membrane proteins gp91phox and p22phox, form a non-active heterodimer known as flavocytochrome b558 (53). If cells are exposed to microorganisms or other mediators of inflammation, the cytosolic proteins p67phox, p47phox, and p40phox, are transferred to the plasma membrane where they associate with flavocytochrome b558. This interaction leads to an activated NADPH oxidase complex and resulting production of superoxide anions (50). The active complex converts NADPH into NADP+ and generates two electrons and a proton. The electrons then are transported through the NADPH oxidase catalytic subunit (gp91phox) to the final electron acceptor, O2 (50). In essence, the activity control of Nox1, Nox2, and Nox3 is primarily achieved through the cytosolic proteins.
Nox2 and Nox4 have been suggested to play a role in diabetes. Nox2 is among one of the most widely distributed Nox isoforms. It has been described in a large number of tissues including thymus, small intestine, colon, spleen, pancreas, ovary, placenta, prostate, and testis (6). Nox2 can also be found in neurons, cardiomyoctes, skeletal muscle myocytes, hepatocytes, and endothelial cells (6). In phagocytes, Nox2 localizes to both intracellular and plasma membranes. In non-phagocytic cells the localization of Nox2 varies depending on the cell type. In smooth muscle cells, Nox2 is found to colocalize with the perinuclear skeleton and in hippocampal neurons it is suggested Nox2 is localized in the membranes of the synaptic sites (6).

Nox4 is one of the most abundant and extensively expressed NADPH oxidase isoforms with constitutive ROS-generating activity (50). Nox4 shares only 39% identity to Nox2 (6). Nox4 is associated with intracellular compartments and organelles, including the endoplasmic reticulum, mitochondrion, cytoplasm and nucleus (7). Nox4 expression is high in the cerebral vasculature. Because the activity of Nox4 has been thought to be constitutive, transcription to mRNA determines its rate of ROS production. Nox4 is usually co-expressed with both Nox1 and Nox2 but is expressed at higher levels (7). Research also has found that Nox4 may respond to many different agonists linking this oxidase to differentiation, migration, growth, apoptosis, senescence, proinflammatory responses and oxygen sensing (7, 29, 50).

**Nitric Oxide**

Nitric oxide (NO) is another free radical that is a potent vasodilator and can also cause deleterious effects in excess amounts (37). It is generated through
activity of nitric oxide synthase (NOS) and can be calcium-dependent or constitutively expressed depending on the isoform (47). NO modulates cellular respiration and is also believed to act as a neurotransmitter (47). NO can also act as an antioxidant, however when NO and O$_2$ combine they form the highly reactive peroxynitrite that attacks and inhibits proteins and lipids causing it to become a prooxidant (47). Endothelial nitric oxide (eNos) is responsible for vascular NO production (14).

*Mitochondria*

Another source of ROS production is the mitochondrial electron transport chain. The electron transport chain oxidizes the reduced cofactors, FADH2 and NADH, by transferring electrons to oxygen through a series of steps. The free energy from oxidation-reduction reactions in the electron transport chain is used to form a membrane pH gradient across the inner mitochondrial membrane that provides energy for ATP synthesis (33, 37). During this process, some molecular oxygen is reduced thus forming the superoxide.

The electron transport chain is made up of four complexes, complex I, II, III, and IV (37). Under normal conditions, NADH and pyruvate are generated during glycolysis. NADH donates electrons to the mitochondrial electron transport chain while pyruvate enters the citric acid cycle and produces NADH and FADH. Both NADH and FADH provide the electrons that fuel the electron transport chain and ATP production. NADH derived from glucose oxidation and from the citric acid cycle donates electrons to complex I while FADH2 donates its electrons to complex II. Both complex I and II then transfer electrons to ubiquinone through Fe-S centers
Complex II also has a heme group (heme \( b \)) that doesn’t participate in the electron transfer but instead protects against the formation of ROS by electrons that are not transferred to ubiquinone (36). Complex III couples the transfer of electrons from ubiquinone to cytochrome \( c \). Cytochrome \( c \) is a soluble protein of the intermembrane space of the mitochondria and once it accepts the electron from complex III, it moves to complex IV, also known as cytochrome oxidase, which carries electrons from cytochrome \( c \) to molecular oxygen (33, 36, 37).

As the electrons are transferred through the electron transport chain the energy is used to shuttle protons across the membrane. For each electron that passes through complex IV, a proton is pumped outward into the intermembrane space adding to the proton transport of complexes I and II (33). This creates a voltage across the inner and outer membrane of the mitochondria and drives ATP synthesis (37). In hyperglycemic conditions, the number of substrates entering the citric acid cycle is greatly increased and consequently the number of reducing equivalents donating electrons to the electron transport chain is also increased (37). Once the electron transport chain reaches a threshold voltage across the membrane the electrons begin to back up at complex III. These electrons are then donated to molecular oxygen, which in turn results in an increase in mitochondrial superoxide production. (37)

**Antioxidants**

Organisms are equipped with complicated endogenous antioxidant defense systems to eliminate deleterious levels of ROS (53). There are both enzymatic and non-enzymatic defense mechanisms. The non-enzymatic defense mechanisms
include glutathione, ascorbate (vitamin C), carotenoids, vitamin E, and various phenylpropanoid derivatives (8, 53). The enzymatic defense mechanisms include glutathione reductase, superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) (8, 53). These enzymes catalyze the synthesis and recycling of antioxidant molecules or can directly catalyze the removal of free radicals from cells (8, 53).

During normal physiological concentrations, ROS is rapidly removed by these endogenous antioxidant systems. However, when the ROS production out-weighs the antioxidant defense systems, oxidative damage may occur due to accumulation of oxidation products.

Summary

Diabetes is becoming increasingly prevalent. Cerebral edema is a harmful and potentially fatal complication of diabetes. Hyperglycemia has been hypothesized to increase ROS production with evidence of increased oxidative stress markers during episodes of hyperglycemia. Cell swelling during brain edema also results in increased ROS production. The harmful effects of diabetes have been associated with ROS production in many tissues and cell types. However, little is known about this potential pathological process in the brain. This study will examine ROS production influenced by interactions between cell swelling and high glucose conditions in a glial cell line.
SPECIFIC AIMS
II. Specific Aims:

Reactive oxidative species (ROS) are necessary for normal physiological function but increased amounts can have detrimental effects. During hyperglycemia the rate of ROS production is greatly increased potentially causing cell injury. The rate of ROS production also is increased in many cell types during osmotic swelling. Thus, I hypothesize that when cells are treated with high glucose media and then swollen by exposure to hypoosmotic conditions, there will be an additional increase in ROS production. I will directly test this hypothesis by examining mechanisms of hyperglycemic-induced ROS production and hypoosmotic-induced ROS production in rat glioma C6 cells.

Specific Aim I

I hypothesize that C6 cells treated with high glucose media increase their ROS production. I will incubate C6 cells in high glucose media for 24 hours and then measure the rate of intracellular ROS production using a fluorescence imaging system and ROS-sensitive dyes.

Specific Aim II

I hypothesize that C6 cells treated with high glucose media and then swollen in hypoosmotic conditions increase their ROS production. I will expose high glucose treated cells to hypoosmotic conditions and then measure the rate of intracellular ROS production using a fluorescence imaging system and ROS-sensitive dyes. I also will examine the mechanism of ROS production using a pharmacological agent to block the activity of NADPH oxidases.
These studies will examine the relationship between ROS production, hyperglycemia and volume regulation. It will further our understanding of the mechanisms of ROS production in hyperglycemic glial cells and their potential to produce cell injury.
MATERIALS AND METHODS
III. Materials and Methods

Materials

5-(and-6-)dichlorodihydrofluorescein diacetate (DCFDA), calcein AM and dihydroethidium (DHE) came from Molecular Probes (Eugene, OR). Trypsin, horse serum, fetal bovine serum, penicillin, streptomycin, and trypan blue came from Invitrogen (Grand Island, NY). Ham’s F-12 Kaighn’s medium, L-glutamine and chemicals including carbenoxolone, hydrogen peroxide, sodium bicarbonate, diphenyleneiodonium (DPI), BCA protein assay kit, mammalian cell lysis kit, and glucose came from Sigma-Aldrich (St. Louis, MO) or Fischer Scientific (Hanover Park, IL). 12 mm diameter coverslips came from Fischer Scientific. Nox2 and Nox4 antibodies came from Abcam (Cambridge, MA). Mini-Protean TGX precast gels came from BioRad.

Glial Cultures

C6 rat glioma cells obtained from American Type Culture Collection (Manassas, VA) were plated onto 60 mm Petri dishes or 25 cm² flasks with 3 ml of growth medium consisting of Ham’s F-12 Kaighn’s medium (Ham’s F-12K) with 2 mM L-glutamine plus 15% horse serum, 2.5% fetal bovine serum, 100 units/ml of penicillin, and 100 µg/ml of streptomycin. Ham’s F-12K medium was made from powder and was supplemented with 1.5 g/L sodium bicarbonate according to the manufacturer’s instructions. Cells were replated weekly at a 1:100 dilution or a 1:50 dilution. To remove the cells from the flask for replating, we first replaced the growth medium with 3 ml of 0.25% trypsin to loosen the cells from the culture surface. When cells had been isolated and were floating in the trypsin solution (15
min) we added an equal volume of complete medium to inactivate the trypsin. The resulting cell suspension was centrifuged for 5 min and the supernatant medium with trypsin was decanted and discarded. The cell pellet then was suspended in fresh growth medium for plating onto culture flask and dishes. C6 cells were plated at the same density in flasks and dishes. The dishes contained 12 mm diameter glass coverslips, which had been cleaned by soaking in nitric oxide for 24 hours, followed by multiple washings with distilled water. Once washed, the coverslips were sterilized for 30 minutes in an autoclave. Cells grew equally well on glass and plastic surfaces. Cells were maintained at 37 °C in an atmosphere of room air with 5% CO₂.

The growth medium was changed 3-4 days after plating. These cells were grown in normal glucose medium and were defined as normoglycemic cells. Some cells were grown in high glucose (26 mM) C6 cell medium and were defined as hyperglycemic cells. Three days after replating cells, growth medium was removed and replaced with high glucose medium for 24 hours before being used for experimental procedures.

Cell Fluorescence Imaging

The set up of the fluorescence imaging system is shown in Figure 1. The microscope used to capture the cell images was an Olympus 55B reflected epifluorescence system. The microscope was connected to the perfusion solutions by a tube that flowed through a peristaltic pump and pre-heater before perfusing into the cell chamber. A second tube removed the perfusion solution from the cell chamber and into a waste bottle. ImagePro 6.3 was used to capture the cell images with the microscope camera while controlling a shutter, which limited the amount
of time the cells were exposed to the fluorescence excitation light. Typically, a 30-minute time-lapse experiment was used with the exposure time and time interval between exposures independently adjusted.

Calibration and adjustment of the perfusion system was performed to find a rate of flow for the PBS solution that would limit coverslip movement and allow a quick change of perfusion solution. I adjusted flow rate to 1ml/minute for all the experiments. A selector valve permitted a rapid change of the perfusion solution from isosmotic PBS to hypoosmotic PBS. To determine the time it took to completely change the solution in the chamber I used a perfusate solution of trypan blue and a clear perfusate solution. I took images periodically to see when the clear solution was removed and when the colored solution filled the chamber. With these experiments, I found that 2 minutes was sufficient to replace the solution in the chamber.

To determine the shutter exposure time that would provide sufficient signal with minimal bleaching of the fluorescent dye calcein was loaded into the 1321N1 (human astrocytoma) cells. Cells were loaded by incubating a coverslip with isosmotic PBS containing calcein AM at 10-50 μM. The isosmotic PBS consisted of 137 mM NaCl, 2.7 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 3.2 mM Na₂HPO₄, 0.5 mM KH₂PO₄, and 5.5 mM glucose (290 mOsm). The osmolarity of the PBS was measured by a vapor pressure osmometer and adjusted by adding a volume of 3 M NaCl. Cells loaded with calcein AM were perfused with isosmotic PBS for 5 minutes. Images were acquired every 30 seconds.

*Measurement of ROS production*
To evaluate the production of ROS, we measured the fluorescence of an exogenous intracellular fluorescent dye in individual cells. Two fluorescent dyes were used in the experiments described here, 10 µM 2′,7′-dichlorofluorescein (DCF) and 5 µM dihydroethidium (DHE). To load cells with DCF, DCFDA was added to the cells and then incubated for 20 minutes prior to performing fluorescence measurements while DHE was continuously perfused onto the cells throughout the experiment. After diffusion into the cell DCFDA is deacetylated by cellular esterases to a non-fluorescent compound which can later be oxidized by ROS into 2′,7′-dichlorofluorescein (DCF). DCF is highly fluorescent and can be detected with maximum excitation and emission wavelengths of 495 nm and 529 nm respectively (1). In the presence of $O_2^-$, DHE is oxidized to 2-hydroethidium (EOH) and intermediate products. These intermediate products can react with $\cdot$OH or $H_2O_2$ to form ethidium (E·) to a much lesser extent. Whereas E· fluorescence is measured at excitation of 500–530 nm and emission of 590–620 nm, the EOH fluorescence is measured at an excitation and emission wavelength of 480 nm and 567 nm, respectively (23). All cells received the same concentration of DHE. This allowed us to compare the rates of ROS production for cells receiving different treatments.

The coverslip with C6 cells attached was secured in the chamber by a horseshoe shaped wire. This pushed the coverslip down into the chamber to stabilize it. Isoosmotic PBS and hypoosmotic PBS at 35 °C perfused the cells. Hypoosmotic PBS was identical to isoosmotic PBS but with a lower concentration of NaCl to achieve an osmolarity of 195-200 mOsm. In a typical experiment cells were first perfused with isoosmotic PBS for 15 minutes and then perfused with
hypoosmotic PBS for 15 minutes. PBS solutions generally had the same glucose concentrations as media that cells were incubated in.

*Analysis of Fluorescence Intensity*

For analysis of the cellular fluorescence intensity, I selected 6-8 cells that represented the majority of the cells in terms of their shape and initial brightness. A region of interest (ROI) was defined around the whole image of each cell. Using ImageJ 1.43v, fluorescence intensities were measured in each ROI. I then found the rate of change of fluorescence intensities of each cell over various periods during isoosmotic and hypoosmotic PBS exposure. The ratio of the rate of change in fluorescence intensity for each cell in hypoosmotic PBS over the rate of change in fluorescence intensity for the cell in isoosmotic PBS was compared and these ratios averaged to obtain one value for each coverslip. In experiments evaluating whether ROS was produced by the NADPH oxidase pathway, 10 µM diphenyleneiodonium (DPI), an inhibitor of NADPH oxidases was present in isoosmotic and hypoosmotic PBS.

*Evaluation of Cell Death*

Trypan blue stain was used to evaluate cell death in some experiments. C6 cells were grown in high glucose medium for 24 hours. Medium was then removed from dishes and cells were incubated in hypoosmotic PBS containing normal glucose concentrations for 30 minutes. Cells then were returned to high glucose growth medium. This medium then was removed and 3ml of a 1 µM trypan blue in isoosmotic PBS was added to the dishes for 5 minutes. Cells were rinsed and then
images were captured from random areas of each dish. I counted the number of cells stained in the images to determine the percentage of cell death.

*Western Blot*

Western blot experiments were done to measure expression of eNos, Nox2 and Nox4. C6 cells were grown on 60mm dishes for 3 days. Media was changed to fresh media with normal glucose or high glucose for 24 hours. Cells then were lysed with a solution containing 25 mM Tris-HCl at pH 7.4, with 150 mM NaCl, 0.1% SDS, 1% Triton X-100, and 1% deoxycholate plus a cocktail of proteolysis inhibitors. The resulting lysate was frozen at -20° C. Protein was measured in each sample using the BCA method. Gel electrophoresis was run at 90V for 90 minutes and then samples were blotted onto PVDF membranes with 50 mA at 4° C. Once blots were prepared they were probed using antibodies for eNos, Nox2 and Nox4. Intensity of the band densities was analyzed using ImageJ.

*Statistics*

A Kolmogorov Smirnov test of normality was done on the distribution of ratios for each experiment conducted. Ratio values were log transformed for further analysis. The logs of the fluorescence ratios were analyzed by ANOVA. Comparisons of the log ratio values between experimental groups were performed using Student’s t test. Significance was indicated for P < .05.
RESULTS
IV. Results

**Fluorescence Imaging**

The shutter exposure time and the excitation light attenuator settings were adjusted to cause the least amount of bleaching of the cells yet produce sufficient signal. Bleaching is the photochemical alteration of a dye or a fluorophore molecule such that it is permanently unable to fluoresce (41). The exposure time was varied between 0.1 sec and 10 sec with attenuators which blocked 75% or 94% of the excitation light. For all exposure times using the 94% attenuator, the fluorescence light signal was too low for analysis. With the 75% attenuator, a sufficient signal was obtained. Calcein bleaching was not apparent for any exposure time less than 1 sec. To assure experiments were performed with minimal bleaching, the exposure time chosen as optimal was 0.5 sec with an attenuator that blocked 75% of the excitation light beam (Figure 2a and 2b).

**ROS Production**

Initial studies of ROS production were measured with 1321N1 cells following DCF loading in isoosmotic and hypoosmotic PBS (Figure 3). Cells were incubated with 10 µM of DCFDA and then images were taken every 30 seconds while the cells were perfused with PBS as described in Methods. In isoosmotic PBS the rate of change in fluorescence intensity was found to decrease over time. Since cells produce a constant rate of ROS during normal physiological function this suggested that the DCF dye might have been leaking out of the cells or the excitation light was bleaching the dye. To prevent potential leaking of the DCF from 1321N1 cells carbenoxolone, an inhibitor of gap junctions and connexon hemichannels was added.
to the PBS (Figure 3). The rate of change in fluorescence intensity increased and remained constant in isoosmotic PBS and then decreased when hypoosmotic PBS was added suggesting that these cells also were becoming leaky for the dye when swollen or that the dye was becoming bleached. The cell line used for the experiments was then changed to C6 rat glioma cells, a cell line which had previously shown consistent increase in DCF fluorescence intensity in our laboratory.

Experiments were performed to confirm that DCF could detect ROS when loaded into C6 cells (Figure 4). The addition of H$_2$O$_2$ to the isoosmotic perfusate caused an abrupt increase in fluorescence intensity followed by a decrease of intensity towards the end of the study. The initial increase of fluorescence intensity during H$_2$O$_2$ exposure showed that ROS was being detected by the dye. The decrease in the rate of change in fluorescence intensity could be attributed to leakage of the dye due to persistent exposure to H$_2$O$_2$.

Because of the uncertainties of DCF leakage, I changed experimental protocol to use the DHE as a ROS indicator because it is continuously loaded into the cells through the perfusion solution and becomes bound to the nucleic acids after oxidation, thus limiting any potential leaking. Figure 4 shows results from one normoglycemic cell and one hyperglycemic cell. The rate of change in fluorescence intensity of both cells was consistent in isoosmotic and hypoosmotic PBS suggesting there was no leakage of the dye.
Figure 1

Set up of Experiment. Perfusion solution flows through the tubes and then through the selector valves connecting to one tube. The solution then flows through the peristaltic pump, to the pre heater and then to the cell chamber. As more solution enters the cell chamber, the old solution is removed to the waste bottle by another tube. Light from the mercury lamp enters the shutter, which is controlled by the computer and then goes through the microscope to the cell chamber. It is reflected back up through the microscope to the camera where an image is taken. The computer receives and stores the image using ImagePro 6.3.
Figure 2a and 2b

Shutter exposure times that minimized bleaching. Different shutter exposure times were tested for attenuators 6 (blocking 94% of the excitation light) and 25 (blocking 75% of the excitation light). Attenuator 25 and a shutter exposure time of 0.5 sec minimized bleaching and gave sufficient fluorescence intensity.
A.

Normalized Intensity Values Using Attenuator 25

![Normalized Intensity Values Using Attenuator 25]

B.

Normalized Intensity Values Using Attenuator 6

![Normalized Intensity Values Using Attenuator 6]
Figure 3

1321N1 cells loaded with DCF and perfused in the presence or absence of carbenoxolone. Each trace is from a single 1321N1 cell. The cells loaded with DCF were perfused with 35°C isoosmotic and hypoosmotic PBS for 10 minutes with images taken every 30 seconds. The arrow indicates the change of perfusate from isoosmotic PBS to hypoosmotic PBS. The rate of change in fluorescence intensity decreased over time both in isosmotic and hypoosmotic conditions. Carbenoxolone added to the perfusate did not prevent the decrease.
1321N1 Cells with DCF and 1321N1 Cells with DCF plus Carbenoxolone

Rate of Change in Fluorescence Intensity (Arbitrary Units)

Time (Min)
Figure 4

C6 cells loaded with DCF and sequentially perfused with isosmotic PBS solution and isosmotic PBS plus H$_2$O$_2$ (200 μM). Images were taken every 30 seconds. The rate of change in fluorescence intensity increased rapidly with the addition of H$_2$O$_2$ but then reversed towards the end of the exposure. C6 cells were perfused with 35º C isoosmotic PBS for 6 minutes and then perfused with isoosmotic PBS plus H$_2$O$_2$ for another 6 minutes. The arrow indicates the addition of H$_2$O$_2$ to the isoosmotic PBS perfusate. The line represents the linear regression of the fluorescent intensity over the course of the entire experiment.
C6 Cells in Isoosmotic PBS and Isoosmotic-H2O2 PBS with DCF

Addition of Iso-H2O2
Figure 5

DHE fluorescence intensity of C6 cells perfused with isoosmotic and hypoosmotic PBS. C6 cells were incubated in normoglycemic conditions or hyperglycemic conditions for 24 hours. The cells were perfused with isoosmotic PBS plus DHE (5 µM) for 15 minutes and then perfused with hypoosmotic PBS plus DHE (5 µM) for another 15 minutes. The arrow indicates the change to hypoosmotic PBS. Each trace is from a single cell. Images were captured every minute. The linear regression of fluorescence intensities in isoosmotic and hypoosmotic conditions is shown as the straight lines. The lines were calculated using data from time points between 5 min and 15 min for isoosmotic conditions and between 18 min and 25 min for the hypoosmotic conditions. The lines in the graphs are extended beyond these ranges to help visualize the slope changes. The hyperglycemic cell increased its rate of change in fluorescence intensity when perfused with hypoosmotic PBS.
C6 Cell in Isoosmotic and Hypoosmotic Conditions Normal Glucose vs High Glucose with DHE

Rate of Change in Fluorescence Intensity (Arbitrary Units)

Time (Min)

Hyperglycemic

Normoglycemic
The basal rate of change in fluorescence intensity is defined as the fluorescence intensity measured when cells were perfused with isoosmotic PBS. The basal rate of change in fluorescence for isoosmotic normoglycemic cells was 0.43 ± 0.17 arbitrary fluorescence units (a.f.u)/min while the basal rate of change in fluorescence intensity for isoosmotic hyperglycemic cells was significantly higher at 0.71± 0.45 a.f.u/min. The rate of change in fluorescence intensity was unchanged when normoglycemic cells were perfused with hypoosmotic PBS (Figure 6). However, the rate of change in fluorescence intensity increased for hyperglycemic cells when swollen in hypoosmotic PBS to 0.87 ± 0.44 a.f.u/min.

Figure 7 shows the ratios of the rates of change in fluorescence intensity during perfusion in isoosmotic and hypoosmotic PBS for normoglycemic cells and hyperglycemic cells. The ratio of the rates of fluorescence change for normoglycemic cells was not significantly different from 1.0. Hyperglycemic cells showed a significant increase in the rate of change in fluorescence when perfused with hypoosmotic PBS. The average of the ratios for normoglycemic cells compared to that of hyperglycemic cells was statistically significant.

Nox Inhibition

DPI was used to inhibit NADPH oxidase enzymes, a prominent pathway of ROS production. There was no difference in the basal rate of change in fluorescence intensity when normoglycemic cells were perfused with DPI. Similar to the response of swollen normoglycemic cells without DPI exposure, there was no difference in the rate of change in fluorescence intensity when normoglycemic cells perfused with DPI were swollen in hypoosmotic PBS. In contrast, hyperglycemic cells had a lower
basal rate of change in fluorescence intensity when perfused with DPI. There was also a decrease in fluorescence intensity when hyperglycemic cells were perfused with DPI in hypoosmotic PBS (Figure 8). Ratios of the rate of change in fluorescence intensity for hyperglycemic and normoglycemic cells and hyperglycemic and normoglycemic cells perfused with DPI during isoosmotic and hypoosmotic conditions were compared (Figure 9). There was a significant increase in fluorescence intensity in hyperglycemic and normoglycemic cells perfused with DPI.
**Figure 6**

Average rate of change of fluorescence intensity of normoglycemic cells and hyperglycemic cells in isoosmotic and hypoosmotic PBS. The rate of change in fluorescence intensity in isoosmotic and hypoosmotic PBS conditions was found by calculating the slopes of the fluorescence intensities between 10 min and 15 min and between 18 min and 25 min. The slope calculated for every cell on a coverslip then was averaged resulting in 17 data points for isoosmotic exposure and 17 data points for hypoosmotic exposure. The mean ± SEM of these values is shown here.

* Indicates a significant difference compared with normoglycemic cells in the same osmolality PBS.
Average Rate of Change in Fluorescence Intensity for C6 Cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>Rate of Change in Fluorescence Intensity (Arbitrary Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoosmotic</td>
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</tr>
<tr>
<td>Hypoosmotic</td>
<td>0.4</td>
</tr>
<tr>
<td>Normoglycemic</td>
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<tr>
<td>Isoosmotic</td>
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<tr>
<td>Hyperglycemic</td>
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</table>

*Significant difference compared to Normoglycemic condition.
Figure 7

Ratios of the rates of change in fluorescence intensity measured in isoosmotic and hypoosmotic PBS. The rate of change in fluorescence intensities was determined as the slope of the regression line calculated between 10 min to 15 min for isoosmotic conditions and between 18 min to 25 min for hypoosmotic conditions. The ratios of the average slopes measured for cells perfused with hypoosmotic PBS relative to that measured when the cells were perfused with isoosmotic PBS was computed for each coverslip. The relative change in fluorescence intensity for hyperglycemic cells was 39% larger than the relative change in fluorescence intensity for normoglycemic cells.

* Indicates statistical significance between the ratios for normoglycemic cells and hyperglycemic cells
Ratios of Fluorescence Intensity of C6 Cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>Rate of ROS production (Arbitrary Units)</th>
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</thead>
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<td>Normoglycemic</td>
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<tr>
<td>Hyperglycemic</td>
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</table>

*Significant difference between conditions.
Figure 8

The rate of change in fluorescence intensity in isoosmotic and hypoosmotic conditions for cells exposed to DPI. Cells were perfused with isoosmotic and hypoosmotic PBS plus DPI for 30 minutes compared to cells without DPI exposure. There was no difference in the basal rate of change in fluorescence intensity in normoglycemic cells perfused with DPI in hypoosmotic PBS. Hyperglycemic cells had a decreased rate of change in fluorescent intensity when perfused with either isoosmotic or hypoosmotic PBS plus DPI.
Average Rate of Change in Fluorescence Intensity of C6 Cells With DPI

Rate of Change in Fluorescent Intensity (Arbitrary Units)

<table>
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<tr>
<th></th>
<th>Isoosmotic</th>
<th>Hypoosmotic</th>
<th>Isoosmotic</th>
<th>Hypoosmotic</th>
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<tbody>
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<td>Normoglycemic</td>
<td>Hyperglycemic</td>
<td>Normoglycemic</td>
<td>Hyperglycemic</td>
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<tr>
<td>Average Rate of Change in Fluorescence Intensity of C6 Cells With DPI</td>
<td>0.30</td>
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Figure 9

Ratios of the rates of change in fluorescence intensity in isoosmotic and hypoosmotic PBS with DPI. The rate of change in fluorescence intensities was determined as the slope of the regression line calculated between 10 min to 15 min for isoosmotic conditions and between 18 min to 25 min for hypoosmotic conditions. The ratios of the average slopes measured for cells perfused with hypoosmotic PBS plus DPI relative to that measured when the cells were perfused with isoosmotic PBS plus DPI was computed for each coverslip.

* Indicates statistical significance between the ratios for normoglycemic cells and hyperglycemic cells
Ratios of Fluorescence Intensity of C6 Cells with DPI

Fluorescence Intensity (Arbitrary Units)

Normoglycemic and DPI

Hyperglycemic and DPI

*
**Hyperglycemic C6 cells Perfused With Normoglycemic PBS**

To model brain edema development during treatment for DKA, additional experiments were done to examine rate of change of fluorescence intensity in hyperglycemic cells when perfused with PBS solutions containing normal glucose concentrations. Figure 10 shows results from a single cell typical of experiments from 6 coverslips. When hyperglycemic cells were swollen in hypoosmotic conditions containing normal glucose concentrations, they experienced a decrease in fluorescence intensity. Trypan blue stain was done to determine if the decreased fluorescence signal for cells during hypoosmotic exposure with normal glucose was due to cell death. The results demonstrated cell death was less than 10% in the hyperglycemic cells exposed to normal glucose hypoosmotic PBS (Figure 11)

**Western Blot Analysis**

To determine if endothelial Nos contributed to the increased production of ROS in hyperglycemic cells, western blot analysis was done. Figure 12 shows that when intensity of the bands for the expression of eNos was measured, both normoglycemic cells and hyperglycemic cells had the same density. These intensities represent an average of three dishes from each condition. Image 1 shows an image of the western blot film. The first row of bands was used to measure the intensity of eNos.
Figure 10

Fluorescent intensities of a single hyperglycemic cellperfused in normoglycemic isoosmotic PBS and hypoosmotic PBS. C6 cells were incubated in high glucose media (26 mM) for 24 hours and then perfused with normal glucose (5 mM) hypoosmotic PBS plus DHE for 30 minutes. The arrow indicates the change to hypoosmotic PBS. The cell showed decreased fluorescence intensity when osmotically swollen.
Hyperglycemic C6 cell in 5mM Isoosmotic and Hypoosmotic PBS

Fluorescence Intensity (Arbitrary Units)

Time (Min)
Figure 11

Trypan blue stain of hyperglycemic C6 cells. Cells incubated in high glucose medium for 24 hours were incubated in normal glucose (5 mM) hypoosmotic PBS for 30 minutes. Examples of three studies are shown here. Cell death was less than 10%. The arrows show examples of stained dead cells.
**Figure 12a and 12b**

Western blot analysis for expression of eNos in normoglycemic and hyperglycemic cells. Cells grown in normoglycemic and hyperglycemic conditions were probed to determine expression of endothelial Nos. A: The blot showed high molecular weight bands, which represented eNos expression. B: Intensity of bands was measured. There was no difference in expression of endothelial Nos for normoglycemic cells and hyperglycemic cells.
A. 

B. 

**eNos Expression Intensity in Normoglycemic and Hyperglycemic C6 Cells**

![Graph showing eNos expression intensity in Normoglycemic and Hyperglycemic C6 Cells](image)
DISCUSSION
V. Discussion

The studies show that hyperglycemic C6 cells have a higher basal rate of ROS production than normoglycemic C6 cells. In addition hyperglycemic C6 cells exposed to hypoosmotic conditions increase their rate of ROS production while no increase in ROS production is observed for normoglycemic cells swollen in hypoosmotic conditions. Blocking NADPH oxidase with DPI decreases the basal rate of ROS production in hyperglycemic cells but does not inhibit the hypoosmotic induced increase in ROS production observed in hyperglycemic cells. The increase in ROS production of hyperglycemic cells is not due to increased expression of eNOS. Hyperglycemic cells perfused with normoglycemic hypoosmotic PBS have decreased ROS production indicating potential cell injury.

Optimal conditions for measuring fluorescence in C6 cells

The first few experiments done were used to establish a proper method of measuring and collecting data for the experiments. The cell line used for these initial experiments was the 1321N1 cell line of human astrocytoma cells. I used this cell line because they were a model for glial cells and were derived from human tissue. By loading cells with the fluorescent dye calcein, I found the shutter exposure time and attenuator that would allow for the least amount of bleaching of the dye while giving a measurable signal.

Measurements of ROS production in isoosmotic PBS and hypoosmotic PBS then were performed using 1321N1 cells loaded with DCF. ROS is always being made in cells as part of their physiological functions (2). Others have found in a
variety of cells exposed to hypoosmotic conditions they increase their production of ROS (17). Thus, I expected to find a constant basal rate of ROS production and an enhanced rate of ROS production in the cells when swollen in hypoosmotic PBS. When I exposed the normoglycemic 1321N1 cells to either isoosmotic or hypoosmotic PBS, there was often a decrease in ROS production. This suggested that the 1321N1 cells became leaky when swollen causing the DCF to leave the cell. Carbenoxolone, which had been used previously in our lab, was added to the perfusate to decrease the potential for leaking gap junction hemichannels (49). With carbenoxolone, normoglycemic cells showed increased fluorescence intensity in isoosmotic PBS but decreased fluorescence intensity when the cells were swollen in hypoosmotic PBS.

Because of the potential leakiness of the 1321N1 cells, I changed to use the C6 cell line because it had been used in previous experiments on cell volume regulation (46) and showed consistent increase in ROS production in our laboratory (J.O personal communication). First H₂O₂ was added to the perfusate to allow me to see if the dye was capable of detecting ROS that was being produced extracellularly. As seen in Figure 2 there was an increase in intensity during isoosmotic PBS treatment and even greater increase when isoosmotic PBS with H₂O₂ was added. The increased fluorescence intensity indicated that the dye was being oxidized. However, there was a decrease in ROS production towards the end of the experiment, which could potentially be attributed to cytotoxicity of H₂O₂. The continuous presence of H₂O₂ can cause damage to proteins, lipids and nucleic acids (48). To avoid potential leakiness of DCF, DHE dye was used as a ROS indicator. DHE
added to the perfusion solution, allowed the dye to continuously load into the cells during the experiment. When DHE crosses the plasma membrane and becomes oxidized by ROS it remains bound to DNA. There was no decrease in fluorescence intensity when DHE was used.

*Hyperglycemic conditions causes increased basal ROS production in C6 cells*

C6 cells produce ROS at a constant rate as a part of normal physiological functioning (5, 12, 38). Figure 4 shows that for a single C6 cell in normoglycemic conditions, there was a continuous production of ROS over time. Following incubation in hyperglycemic conditions however, there was a greater rate of basal ROS production than was observed in normoglycemic cells. Hyperglycemia causes an increased production of ROS production leading to deleterious effects in pancreatic cells, neuronal cells and vascular cells (30, 37, 42). In experiments done by Tramontina et al (2012), C6 cells incubated in high glucose medium as a model for hyperglycemia increased their ROS production by 19% (46). This is consistent with my results of increased ROS production in hyperglycemic cells. In diabetes, hyperglycemia increases neuronal death and activates astrocytes to cause gliosis. Neuronal death has been demonstrated to contribute to the neuropathy associated with diabetes (48). Diabetic neuropathy is a common complication of both type 1 and type 2 diabetes affecting over 90% of diabetic patients. Increased enhancement of the polyol pathway activity and the NADPH oxidases during prolonged hyperglycemia are linked to the primary causes of diabetic neuropathy (4). My results showed that the NADPH oxidase pathway seems to be activated when glial cells are incubated in hyperglycemic conditions. Inhibiting the NADPH oxidase
pathway lowered the rate of ROS production in hyperglycemic cells to the same rate of ROS production seen in normoglycemic cells. The polyol and NADPH oxidase pathways when activated cause excess amounts of superoxide to be made overpowering the antioxidant effects of cells, thus causing oxidative damage. Oxidative damage of tissues caused by hyperglycemia not only leads to neuropathy but can also lead to retinopathy, nephropathy and damage to critical blood vessels potentially causing peripheral arterial disease (53).

**Osmotically swollen hyperglycemic and normoglycemic cells in hypoosmotic PBS**

When normoglycemic cells were osmotically swollen there was not a significant change in ROS production (Figure 9). This refuted my hypothesis that there would be a significant increase in ROS production during perfusion with hypoosmotic PBS. Many studies show an increase in ROS production in glial cells during hypoosmotic conditions. Reinehr et al. (39) showed an increase in ROS production when primary cultured astrocytes were swollen by hypoosmotic conditions. Mojtabahzadeh et al. (32) showed that traumatic brain injury clinical patients had high concentrations of ROS in their venous blood after the occurrence of cerebral edema. Compared to a group of healthy volunteers with no known psychiatric or neurological disorders, traumatic brain injury patients had significantly higher serum levels of ROS (32). Haussinger and Gorg (20) also showed increased generation of oxidative/nitrosative stress when astrocytes were swollen. This difference in results could be due to the C6 cells and their antioxidant capacities in hypoosmotic conditions or my methods of using DHE instead of using
DCF like these previous studies. Further research has to be done to evaluate my results.

Compared to basal ROS production of hyperglycemic cells, swollen hyperglycemic cells generated a greater amount of ROS. Increased production of ROS in hyperglycemic cells is seen in episodes of DKA (16, 17, 18, 22). Increased ROS production can cause harmful effects to patients. Prolonged DKA can cause a decrease in white matter and grey matter volumes (14). Alterations in attention and memory are also associated with prolonged DKA (14). Earlier diagnosis of DKA could limit these neurological consequences.

*Hyperglycemic cells in normoglycemic PBS*

Hyperglycemic cells perfused with normoglycemic PBS showed a decrease in fluorescence intensity when the cells were swollen. The initial increase during perfusion with isoosmotic PBS and initial period of perfusion with hypoosmotic PBS shows that the cells were making ROS. The subsequent decrease in fluorescence suggested the cells were leaking the ROS indicator dye. In DKA treatment, IV rehydration and administration of insulin is the main form of treatment currently used (17). Excessive rates of fluid administration have been recently discussed as being responsible for exacerbating cerebral edema injury due to DKA (17). It has been hypothesized that rapid fluid infusion with rapid changes in serum osmolality and glucose might lead to brain swelling (17,18). My experiments using trypan blue to stain dead cells were done to determine if the fluorescence intensity was decreasing due to cell death. However, we found the cultures had a very small percentage of death after the normoglycemic hypoosmotic exposure. These cells
were likely not to be dying from exposure to the hypoosmotic PBS despite the
decrease in fluorescence signal intensity due to apparent cellular injury. Further
study with this model system is needed to provide more information about the
mechanism of the observed decrease in fluorescence intensity.

*NADPH oxidase as a mechanism for ROS production*

The NADPH oxidase enzymes Nox2 and Nox4 are a source of ROS production.
Nox enzymes, as highly regulated membrane-associated protein complexes, catalyze
the reduction of oxygen to the superoxide anion. Excess superoxide causes damage
to cells and is specifically seen in hyperglycemic conditions \(22, 39\). Experiments
done by Ma et al. \(31\), found that DPI decreased Nox4 activity and ROS production
in endothelial cells. Also, increased Nox2 activity was seen during cellular damage
and oxidative stress after acute brain injury in neurons and microglia. This activity
was decreased when Nox2 was inhibited \(27\). To determine if this mechanism was
present in C6 cells, I used a pharmacological agent, DPI, that inhibits the activation of
these oxidases. Figure 8 shows that in hyperglycemic cells there was a decreased
rate of ROS production in isoosmotic PBS when cells were treated with DPI. These
results show that the NADPH oxidase pathway is a mechanism of increased ROS
production in hyperglycemic C6 cells.

When comparing the ratio values of normoglycemic cells and normoglycemic
cells perfused with DPI, there is an increase in ROS production in the
normoglycemic cells perfused with DPI. The potential source of the increase in ROS
production could be the electron transport chain. Inhibition of the NADPH pathway
by DPI could unmask small increases in ROS production by the electron transport
chain. Future experiments to inhibit the electron transport chain can determine whether this mechanism increases ROS production in response to swelling.

Nos is increased in the arteries of diabetic rats (47). Thus, I measured the expression of the ROS producing enzyme eNos using western blots to determine if the enzyme was responsible for the increased rate of basal ROS production in hyperglycemic cells as compared with normoglycemic cells. Bands were seen in both normoglycemic cells and hyperglycemic cells for eNos. While both normoglycemic and hyperglycemic cells expressed eNos, the bands were the same density for both treatments. Thus eNos expression was not increased in hyperglycemic cells and so did not effect the increased ROS production seen following hyperglycemic exposure.
SUMMARY AND CONCLUSION
VI. Summary and Conclusions

C6 glioma cells grown in normoglycemic conditions had no change in ROS production when swollen in hypoosmotic PBS. C6 glioma cells grown in hyperglycemic conditions had an increase in basal ROS production and showed a substantial increase in ROS production in hypoosmotic PBS. Increased ROS production can be responsible for CNS damage in diabetes and DKA. NADPH oxidase inhibitor decreased basal ROS production of hyperglycemic cells but had no effect on basal ROS production of normoglycemic cells. Thus, a NADPH oxidase pathway is involved in increased basal ROS production in hyperglycemic cells. However, NADPH oxidase is not involved in the increased ROS production observed in hypoosmotic conditions. Swollen normoglycemic and hyperglycemic cells increased their overall ROS production when perfused with a NADPH oxidase inhibitor.

Western blots probed for eNos showed that there was no change in expression of this ROS producing enzyme following growth in hyperglycemic conditions. Hyperglycemic cells swollen in hypoosmotic PBS, a model for DKA treatment, showed that a dramatic change from a hyperglycemic environment to a normoglycemic environment could result in cell lysis or plasma membrane leakiness. This data suggests that in a clinical setting when treating episodes of DKA, a quick change in glucose concentrations could also be detrimental for the cells.
REFERENCES
VII. References


31. Ma Y, L.W., Yin Y, Li W, AST IV inhibits H2O2-induced human umbilical vein endothelial cell apoptosis by suppressing Nox4 expression through the TGF-


