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Effects of NOX-1 Inhibition on Real-Time Blood Nitric Oxide and Hydrogen Peroxide in Acute Hyperglycemia

Ashley Mawhinney
Philadelphia College of Osteopathic Medicine, ashleymaw@pcom.edu

Philadelphia College of Osteopathic Medicine

The Graduate Program in Biomedical Sciences

Department of Bio-Medical Sciences

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A Thesis in Cardiovascular Disease

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We, the undersigned, duly appointed committee have read and examined this manuscript and certify it is adequate in scope and quality as a thesis for this master's degree. We approve the content of the thesis to be submitted for processing and acceptance.

_____

Qian Chen, Ph.D., Thesis Advisor
Research Assistant Professor
Department of Bio-Medical Sciences

_____

Cathy J. Hatcher, Ph.D., Thesis Committee Member
Assistant Professor
Department of Bio-Medical Sciences

_____

Charlotte Greene, Ph.D., Thesis Committee Member
Professor
Department of Bio-Medical Sciences

_____

Marcus Bell, Ph.D., Program Director, Research Concentration
Master of Science in Biomedical Sciences
Professor
Department of Bio-Medical Sciences

Abstract

Hyperglycemia has been associated with vascular endothelial dysfunction in part by a reduction in nitric oxide (NO) production and increased oxidative stress (e.g., increased superoxide (SO) and hydrogen peroxide (H_2O_2). Endothelial-derived NO can be significantly reduced by increased SO/H_2O_2 in part by the activation of NADPH oxidase during hyperglycemia. Of the 7 NADPH oxidase isoforms, NADPH oxidase isoform 1 (NOX1) is mainly expressed in the vasculature and may play a major role in hyperglycemia induced oxidative stress and vascular endothelial dysfunction. This hypothesis was tested by measuring blood NO and H_2O_2 levels in real time via NO and H_2O_2 microsensors inserted into femoral veins of rats. Hyperglycemia (e.g., 200 mg/dl) was maintained by an i.v. infusion of 30% glucose solution for 3 hours with or without a selective NOX1 inhibitor, ML171. Hyperglycemia for 3 hours resulted in significantly higher blood H_2O_2 levels (3.06±0.4 μM, n=9) compared to the saline infused control ($P<0.01$, n=9). By contrast, ML171 (1 and 5 μM) reduced hyperglycemia-induced H_2O_2 levels by 1.86±0.61 μM ($P<0.05$, n=8) and 4.85±1.02 μM ($P<0.01$, n=5) respectively relative to the hyperglycemic control after 3 hours. Hyperglycemia also significantly reduced blood NO levels (101.41±10.91 nM, n=8) compared to the saline control ($P<0.01$, n=8) after 3 hours. By contrast, ML171 (1 and 5 μM) significantly attenuated the hyperglycemia induced decrease in blood NO levels and increased blood NO levels by 68.48±12.67 nM ($P<0.01$, n=5) and 85.95±12.67 nM ($P<0.01$, n=5) respectively relative to the hyperglycemic control, at the end of experiment. Our results indicate that NOX1 activation may contribute to hyperglycemia-induced oxidative stress and NO reduction. Furthermore, inhibition of NOX1 may mitigate the deleterious effects of hyperglycemia.

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Acknowledgments

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Cathy J. Hatcher, Ph.D., Assistant Professor of Department of Bio-Medical Sciences, Philadelphia College of Osteopathic Medicine, Thesis Committee Member

Charlotte Greene, Ph.D., Professor of Department of Bio-Medical Sciences, Philadelphia College of Osteopathic Medicine, Thesis Committee Member

Dr. Marcus Bell, Director of M.S., Professor of Bio-Medical Sciences, Philadelphia College of Osteopathic Medicine

PCOM Director and Staff of Laboratory Animal Resources

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Center for Chronic Disorders of Aging, Division of Research and the Department of Bio-Medical Sciences, Philadelphia College of Osteopathic Medicine

Introduction

Chronic hyperglycemia in diabetic patients is causally related to micro- and macro-vascular complications. Diabetic patients can have decreased wound healing, decreased immune function, increased oxidative stress, vascular endothelial dysfunction, and increased inflammatory factors[1]. Uncontrolled hyperglycemia can lead to arterial remodeling and atherogenesis, which may result in blocked arteries provoking cardiovascular events. Similarly, acute or stress hyperglycemia in non-diabetic patients can lead to increased myocardial infarction size, negative post-surgical consequences, such as infection, increased morbidity, and mortality[2]. However, the vascular damage due to uncontrolled hyperglycemia has not been yet contained in medical practice. Therefore, understanding the underlying cause of these vascular and inflammatory events during hyperglycemia is critical for better clinical management.

Diabetes Mellitus

Diabetes mellitus is a medical condition that has become an epidemic and a public health concern in recent years. The International Diabetes Federation estimates the current prevalence rate of 9.3% will be doubled by 2035[3]. The American Diabetes Association confirms that 1.4 million Americans are diagnosed every year and 86 million Americans have prediabetes[3]. The CDC reports Diabetes is the 7th leading cause of death, recording over 75,000 deaths in 2014[4]. The expert committee on the diagnosis and classifications for diabetes mellitus criteria of

diagnosis is having hyperglycemia (high blood glucose) of or above 126 mg/dL while fasting for 8 hours due to insulin deficiency (type I) or resistance (type II) on more than one occurrence[5]. Resistance to insulin is usually due to obesity or association with a metabolic syndrome. The normal range for blood glucose levels are between 70 and 100 mg/dL. Key complications of Diabetes are micro- and macro-vascular complications, such as hypertension, cardiovascular disease, blindness, kidney disease, and lower-limb amputation[1]. In 2003-2006, cardiovascular disease death rates were 1.7 times higher among patients with diabetes than without[4]. In 2010, the CDC also had confirmed that hospitalization rates for heart attacks and strokes were 1.8 and 1.5 times more likely in patients with diabetes respectively[4]. The CDC also reported that from 2005-2008, 4.2 million (28.5%) adult diabetes patients had diabetic retinopathy and 4.4% had advanced retinopathy, which could lead to severe vision loss[4]. Moreover, diabetes is responsible for 44% of all kidney failure cases in 2011[4]. Finally, 60% of non-traumatic lower limb amputations occurred in patients diagnosed with diabetes[4].

Acute Hyperglycemia

Acute hyperglycemia, otherwise known as stress hyperglycemia is defined as a condition in which blood glucose levels are above 126 mg/dL temporarily, without a known history of diabetes[5]. It is typically due to surgery, critical illness, sepsis, and/or trauma. It has been reported that stress hyperglycemia occurs 5-30% critical illness cases[6]. Although acute hyperglycemia does not have all the deleterious impacts on a patient as diabetes, it still has been linked to numerous

complications such as a decrease in wound healing and immune function, an increase in myocardial infarctions (MI), post-surgical complications, extended hospital stays, as well as increased mortality rates. Yang et al (2009) determined that acute hyperglycemia increased infarct size (% region at risk) in mice from 34% to 49% (a 45% increase in infarct size)[13]. In 2009, Cruz-Gonzalez et al. found a correlation between non-diabetic patients admitted with hyperglycemia and increase in infarct size. They discovered that hyperglycemia was an independent predictor of infarct size and that hyperglycemia was associated with a larger infarct size[23]. A randomized study by Richards et al (2014) demonstrate that surgical site infections (SSI) in orthopedic injuries were more common in patients with hyperglycemia (21.2%) than without (3.3%)[16]. On a cellular level hyperglycemia has been linked to vascular endothelial dysfunction and subsequent inflammatory responses [10].

Vascular Endothelial Cells and Vascular Endothelial Function

Endothelial cells are a single layer of mesenchymal cells that line body cavities and vascular walls. Endothelial cells specific to the lining of arteries, veins, and capillaries of the skin, heart, lungs, and brain are continuous and nonfenestrated, meaning there is no breaks in the endothelial lining and they lack pores for rapid exchange of molecules[10]. They contain caveolae as well as transient receptor potential channels that assist in regulating fluid and macromolecule passage as well as vascular regeneration, increased permeability and endothelium-derived NO mediated vasorelaxation[10]. These cells produce factors such as

vasodilators (NO, prostaglandins,etc), vasoconstrictors (angiotensin II and endothelin-1), pro and anticoagulants (plasminogen activators and inhibitors, thrombomodulin, von Wilebrand factor,etc), and fibrinolytics and anti-fibrinolytics, growth factors (VEGF), to maintain normal vascular homeostasis or in response to vascular damages, respectively[10]. Their luminal surfaces are capable of regulating trafficking of blood cells through their binding proteins and other molecules. Endothelial cells are also involved in innate and adaptive immunity as well as hemostasis by releasing inflammatory and anti-inflammatory factors (NO), adhesion factors (E-selectin, PECAM-1, VCAM-1, ICAM-1), as well as oxidizing and antioxidizing factors[10]. Vascular endothelial cells play a key role in maintaining vascular homeostasis, vessel regulation, blood clotting and inflammation, as summarized by Favero et al. in figure 1. In particular, endothelial nitric oxide synthase (eNOS) is a key component in vascular endothelial cells.

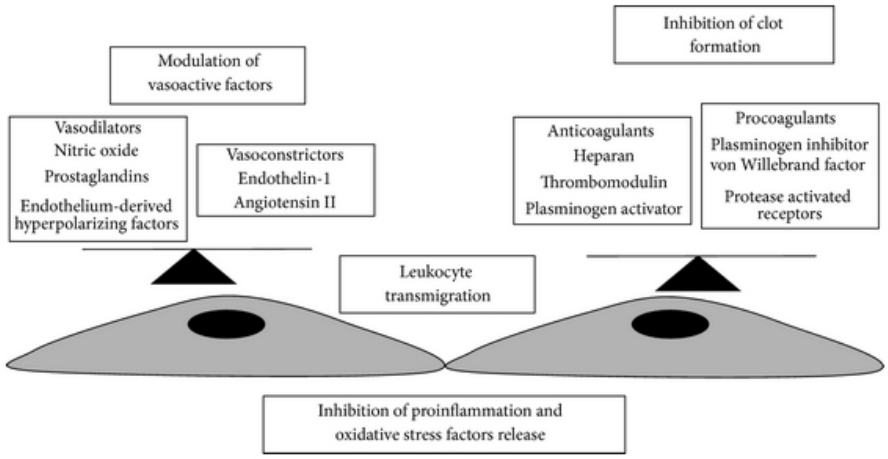

Figure 1: Functions of Endothelial Cells in Physiological Conditions.
Received from Favero, G. et al. Endothelium and Its Alterations in Cardiovascular Diseases: Life Style Intervention. BioMed Research International. 1-28. (2014)

Endothelial nitric oxide synthase (eNOS)

Endothelial nitric oxide synthase (eNOS), is diffusely distributed in the cytoplasm of endothelial cells[10]. Endothelial cells express higher levels of eNOS after stress. The enzyme releases NO which not only prevents inflammation, coagulant signals, and facilitates blood flow, but it is a key factor for maintaining vascular tone and reactivity. NO is also responsible for inhibiting platelet and white blood cell activation and prevents actions of contracting factors such as angiotensin II and endothelin-1 (ET-1)[10]. The eNOS enzyme has two domains with two separate functions, C-terminal reductase domain and an N-terminal oxygenase domain[14]. The C-terminal reductase domain produces electrons by way of FAD/NADPH and the FMN binding sequences it contains. The N-terminal oxygenase domain binds BH_4 and L-arginine and converts L-arginine to L-citrulline, and in the process, produces NO. Both BH_4 and L-arginine are essential for the generation of NO, as demonstrated by early studies showing that depletion of these factors results in SO formation instead of NO, which is described as eNOS uncoupling[14].

eNOS Uncoupling and SO Induced by Oxidative Stress

In pathological conditions, oxidative stress can induce eNOS uncoupling, which shifts its product from NO to SO. This shift from NO to SO also occurs in the N-terminal oxygenase domain, specifically at it's heme center. Recent studies also have also shown evidence that the C-terminal reductase domain produces SO in its FAD/NADPH and FMN binding sites[14]. It has been understood for some time now that in the presence of SO, it can quickly quench nitric oxide (NO) to form

peroxynitrite, another more toxic free radical. Peroxynitrite is responsible for uncoupling eNOS by oxidizing the complex as well as its cofactor BH4, demonstrated in figure 2[18].

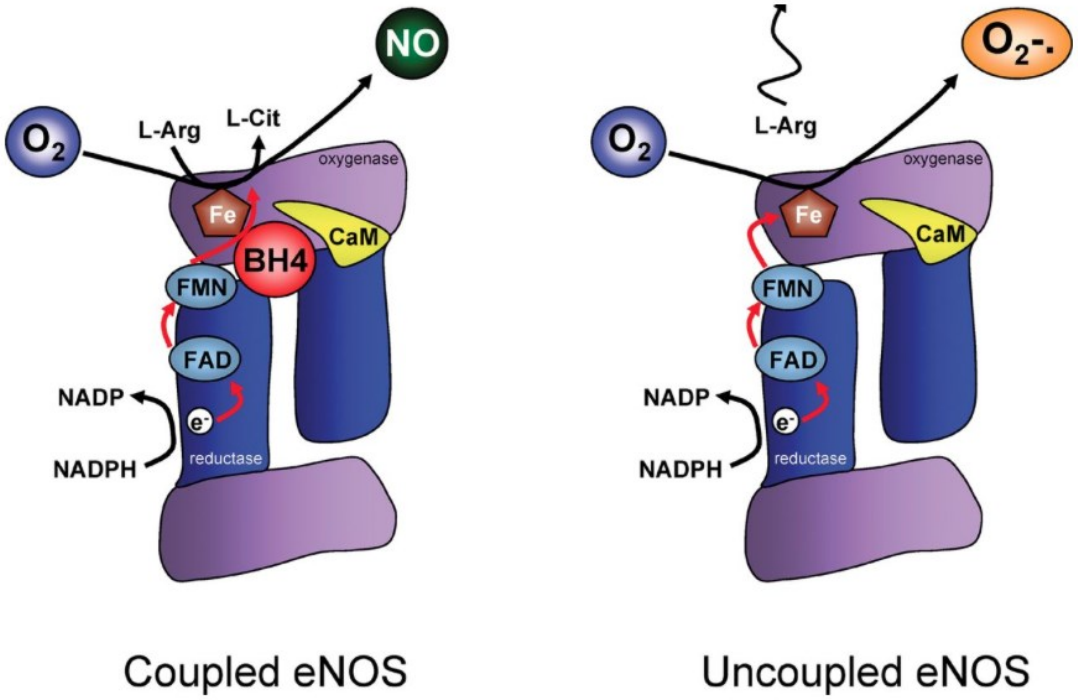

Figure 2: Functions of eNOS, coupled and uncoupled.
Retrieved from Schmidt, T. et al. Mechanisms for the role of tetrahydrobiopterin in endothelial function and vascular disease. Clin Sci (Lond). 113(2):47-63

Youn, et al has also recently demonstrated recently, that Bone Morphogenic Protein 4 (BMP4), which is known for its activity during inflammation, endothelial dysfunction, and atherogenesis, has been linked to eNOS uncoupling[17]. This was demonstrated by increased SO production as well as a decrease in BH_4. They also determined in this same study that NOX1 mediates BMP4-induced eNOS uncoupling, as shown by a diminished BMP4-induced eNOS uncoupling with NOX1 siRNA-transfected cells[17]. The product shift of uncoupled eNOS from NO to SO not only

diminishes NO bioavailability, but also increases SO products, resulting in greater oxidative stress.

Vascular Endothelial Dysfunction Under Hyperglycemic Conditions

Many studies have suggested that chronic and acute hyperglycemia can cause vascular endothelial dysfunction. A meta-analysis conducted in 2015 documented that the effects of hyperglycemia are responsible for decreased macro- vascular function. Studies have demonstrated that macro-vascular function such as endothelium-dependent vasodilation through NO was also decreased due to acute hyperglycemia and suggests that acute hyperglycemia could be responsible for the vascular smooth muscle cell proliferation that is responsible for vascular remodeling, resulting in cardiovascular disease[24].

The deleterious effects of hyperglycemia have been presumed to be initiated by vascular endothelial dysfunction. In type 1 diabetes, endothelial dysfunction is chiefly due to metabolic changes predominantly at the retinal and kidney level, however, in type 2 diabetes, endothelial dysfunction begins before onset[13]. Oxidative stress is believed to be a source of vascular endothelial dysfunction and increased inflammatory factors. Oxidative stress is due to overproduced reactive oxygen species (ROS) overwhelming anti-oxidant mechanisms in the body. ROS that mainly consist of overproduced superoxide (SO) and hydrogen peroxide (H_2O_2). SO can be readily converted to H_2O_2 by superoxide dismutase (SOD). H_2O_2 is much more stable than SO and remains for longer periods in the blood. There are multiple sources of ROS, as previously mentioned, uncoupled eNOS, as well as mitochondria

under dysfunction, xanthine oxidase, and NADPH oxidase[26]. All of these upregulate their production of SO under pathological conditions and in upregulation of NADPH oxidase[26].

As previously mentioned, overproduced SO can quickly quench nitric oxide (NO) to form peroxynitrite resulting in reduced endothelial-derived NO bioavailability. Vascular endothelial dysfunction is not only identified by increased levels of ROS, but also by decreased levels of nitric oxide (NO). Oxidative stress has been linked continuously to the progression of all types of diabetes as well as other pathological conditions. Many sources have concluded that hyperglycemia is the cause of increased ROS, which then leads to other complications[20,21].

NADPH Oxidases

NADPH oxidases are the only enzymes in the body that naturally produce SO/ H_2O_2. NADPH oxidases and also may be a principle source of SO/ H_2O_2 under hyperglycemic conditions that result in eNOS uncoupling. There are a total of 7 isoforms in mammals, NOX -1, -2, -3, -4, -5, and dual oxidase -1/-2. NOX1 through 3 share similar structures consisting of five subunits. Each of these contains two transmembrane units, the NOX unit which is the catalytic unit, and the p22phox unit. They also contain three cytosolic subunits, RAC1 which is a small GTPase responsible for regulation, p47phox/NOXO1 which is responsible for organization, and NoxA1/p67phox which is responsible for activation. Angiotensin II (Ang II) is responsible for the upregulation of vascular NADPH oxidases in physiological conditions through activation of protein kinase C (PKC). Once PKC is activated, it is

translocated to the membrane where it can phosphorylate the p47phox subunit of NADPH oxidase. This phosphorylation causes assembly of the rest of the subunits while also triggers activation of a proto-oncogene, c-Src, to stimulate epidermal growth factor receptor (EGRF), creating a cascade of activations to activate the Rac1 subunit and in turn activate NADPH oxidase[22].

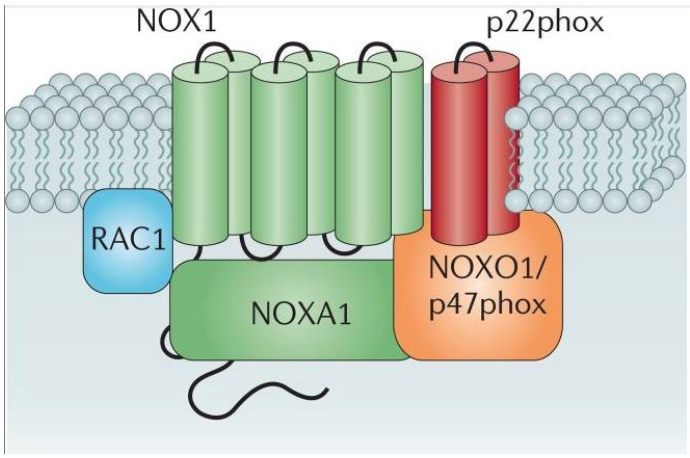

Figure 3: NADPH Oxidase 1 Structure and Composition.
Retrieved from Drummond, G, et al. Combating Oxidative stress in vascular disease: NADPH oxidases as therapeutic targets. 453-471. (2011)

NADPH oxidases are responsible for electron transfer into the electron transport chain with oxygen as the final acceptor. This electron transfer results in the NADPH oxidase releasing superoxide. Some isoforms, such as Nox4, Duox1 and Duox 2 release hydrogen peroxide instead. As previously stated, superoxide(SO) is not as stable as hydrogen peroxide(H_2O_2) and is quickly converted. These enzymes are located in the constitutive cell's arterial walls and are responsible for generation of ROS which leads to oxidative stress. In physiological conditions, NADPH oxidases

(NOX) assist the body with innate immunity, redox-dependent signaling, and production of hormones.

In pathological conditions, NADPH oxidases are responsible for the over production of ROS and oxidative stress in the body. In pathological conditions, an up regulation of NADPH oxidase activation and activity thus leads to an increased and detrimental amount of ROS which causes irreversible damage to the vasculature. NADPH oxidases are activated under hyperglycemic conditions through PKC. During hyperglycemia, the increased levels of glucose are responsible for providing increased levels of diacylglycerol (DAG) through glycolysis. DAG is responsible for activating PKC, and has been accepted as the DAG-PKC pathway. This pathway has been known to regulate endothelial permeability as well as vascular function[5]. Upregulation of PKC in turn upregulates activation of NADPH oxidases.

PKC enzymes, specifically PKC Beta II become activated by hyperglycemia and translocate to the membrane. This translocation as previously stated, puts them in proximity with the NADPH oxidases, causing phosphorylation of the NOX p47phox subunit. Phosphorylation of this subunit stimulates the rest of the NOX subunits to assemble, thus activating the NADPH oxidase.

It has also been recently discovered that NADPH oxidases may also have a role in activation of mitochondrial dysfunction through AngII[22]. Dikalov found that inhibition of NADPH oxidases as well as PKC, prevented of mitochondrial dysfunction[22]. Their studies show that mitochondrial function is associated with NADPH oxidase specific SO. When there is an upregulation of SO and H_2O_2 by

NADPH oxidase, there is an opening of the mitoK_{ATP} channels, which leads to the dysfunction[22]. This was demonstrated by using the NADPH oxidase inhibitors, apocynin and chelerythrine, which prevented mitochondrial dysfunction. Mitochondrial dysfunction leads to decreased membrane potential, respiratory control ratio, and the content of low molecular weight thiols. The results of mitochondrial dysfunction include increased SO and H_2O_2 production on the mitochondrial complex I. When the mitochondria had an increase in SO, there was an increase in cytoplasmic SO as well, suggesting that the mitochondria in turn upregulated NADPH oxidase activity[22]. This further exacerbates the oxidative stress caused by NADPH oxidases, and creates a "cross-talk" of sorts between mitochondria and NADPH oxidases that results in a feed forward mechanism for oxidative stress[22]. The study suggests that this cross talk activity could be mediated by c-Src.. Of the NADPH oxidases, only 1,2 and 4 are expressed in the vasculature, and only 1 and 2 in endothelial cells.

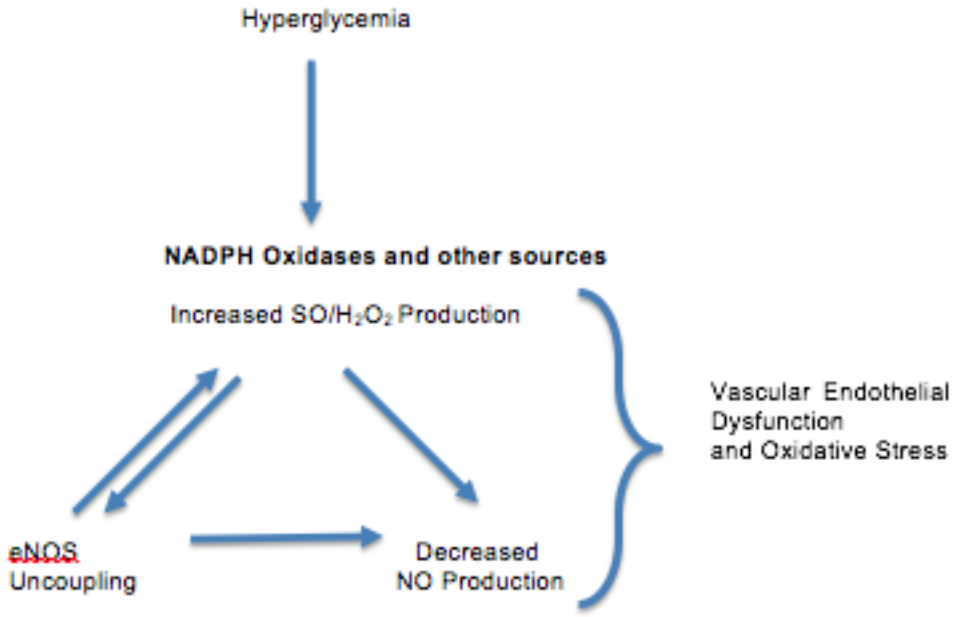

Figure 4. The possible vicious cycle involved in hyperglycemia-induced vascular endothelial dysfunction and oxidative stress.

NADPH Oxidase 1 (NOX1) and NADPH Oxidase 2 (NOX2)

Most NADPH oxidase isoforms are expressed in each cell type of the vascular wall. Of these isoforms, NOX1 and NOX2 are mainly expressed in the vascular endothelial cells and vascular smooth muscle cells (VSMCs). Previous animal studies have determined NOX1 to be the main isoform associated with cardiovascular disease pathology[3]. Previous studies also showed that NOX1 mRNA levels were elevated in the kidney during hyperglycemic conditions as well as deletion of NOX1 attenuating hyperglycemic induced glomerular hypertrophy, mesangial matrix expansion, oxidative stress and cortical senescence, all of which are associated with

renal disease[8]. NOX1 activity has also been linked as the major component of ROS and inflammatory cytokine induced beta cell dysfunction[11].

NOX2, on the other hand, did not show any attenuation of such factors causing diabetic kidney when tested with NOX2 Knock out mice[9]. Previously, our lab has found NOX2 inhibitor, gp91ds-tat, only partially reduced blood H_2O_2 levels under hyperglycemic conditions. However, the role of NOX1 in hyperglycemia induced oxidative stress and vascular endothelial dysfunction is still unclear.

NOX1 Inhibitor: ML171

High-throughput screening has identified a few phenothiazines that exhibit NOX1 inhibition. It was discovered that 2-acetylphenothiazine, otherwise known as ML171, is currently the inhibitor most specific for NOX1, while having only marginal activity with other NADPH oxidases[12]. ML171 was shown to have a 30-fold selectivity over other NOX isotypes. This inhibitor works specifically and directly on the Nox1 subunit, previously mentioned as the catalytic unit of the enzyme. It has been previously discovered that ML171 blocked ROS-dependent formation of extracellular matrix-degrading invadopodia in colon cancers and was reversed when over expression f NOX1 was introduced. ML171 was the selective NOX1 inhibitor as it is the best available at that time.

Hypothesis

It was hypothesized that acute hyperglycemia (200 mg/dL) would increase blood H_2O_2 levels and decrease blood NO levels compared to saline control. By inhibiting NADPH Oxidase 1 phosphorylation/assembly/activation through ML171(2-acetylphenothiazine, MW=241.31 g/mol, Tocris Bioscience), acute hyperglycemia-induced vascular dysfunction would be attenuated. This will be confirmed by decreased blood H_2O_2 levels and increased blood NO levels compared to acute hyperglycemia control.

Methods

NO and H_2O_2 Calibrations

Microsensors (100µm, World Precision Instruments (WPI), Sarasota, FL) for NO and H_2O_2 were calibrated prior to the experiments using known concentrations of NO and H_2O_2 as per manufacturer's suggestion. The electrochemical microsensors membrane detects the NO and H_2O_2 and is analyzed in real-time using the Apollo 4000 Free Radical Anaylzer that allows measurement of multiple species at once and selective poise voltage for ranges needed. Standard curves were used to convert electrochemical changes to concentrations. Standard curves for NO were achieved by soaking the microsensor in 10 mL copper sulfate. The baseline was recorded after at least 10 minutes of soaking, followed by added known amounts (0-200 nM) of standard SNAP solution (100µM- 00022 g S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP) and 0.002 g ethylenediaminetetraaceitic acid (EDTA) were dissolved in 100 mL distilled water) were added and the differences were recorded. To obtain a standard curve for H_2O_2, the microsensor was soaked in 10 mL PBS buffer until stabilized. Following the baseline measurement, known amounts (0-2 µM) of standard (1mM) H_2O_2 was added to develop a calibration formula. The formulas determined from both standard curves were used to convert measurements obtained in real-time to concentrations of blood NO (nM) and blood H_2O_2 (µM).

Animal Preparations

All the procedures used in the animal experiments were approved by PCOM IACUC. Male Sprague-Dawley rats (Charles River: Charles River Kinston, NC), ranging from 275-325g, were used in this study. The rats were anesthetized with pentobarbital sodium 60mg/kg of 50mg/mL injections (i. p.). Maintenance doses were titrated to effect at 30mg/kg (i.p.). Surgical preparations to prepare animal for infusions and monitoring throughout the 180 minutes of the experiment are illustrated in figures 4 and 5.

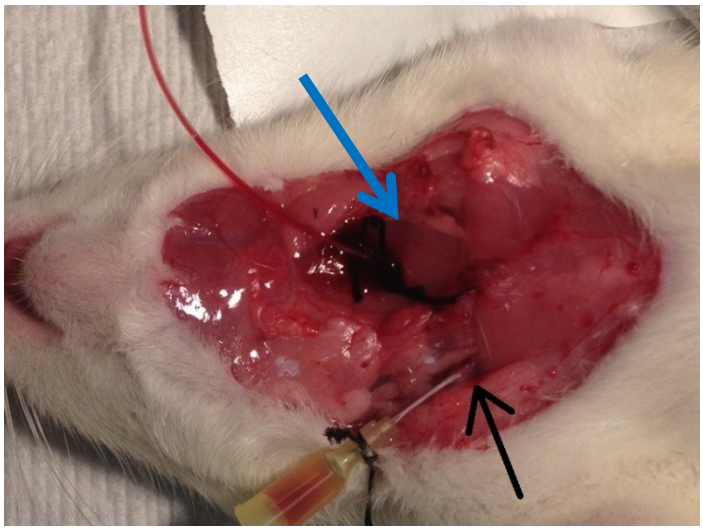

Figure 5. Isolation and catheterization of Jugular vein and Carotid artery.

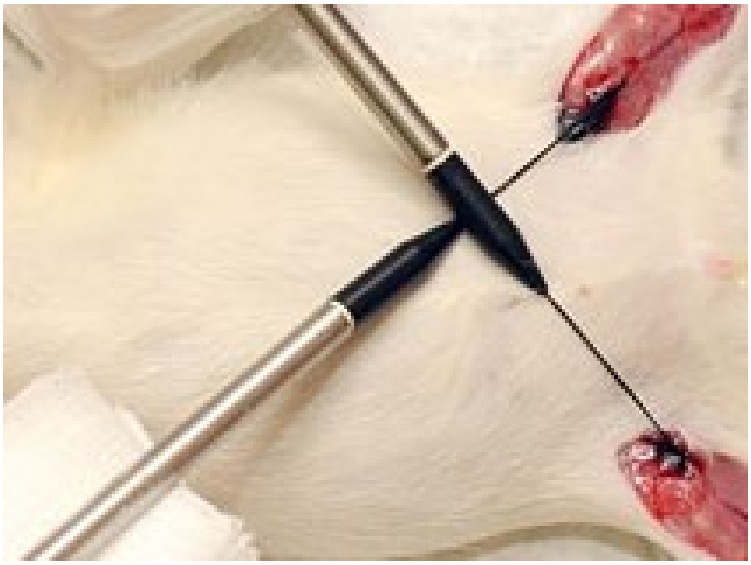

Figure 6. Catheterization of both Femoral veins with inserted microsensors.

The carotid artery was first catheterized in order to monitor status during the experiment (see figure 4-blue arrow). Proper operative conditions were monitored by recording mean arterial blood pressure (MABP) every 20 minutes, throughout the experimental period. Normal MABP levels ranged from 90-130mmHg during the entire experiment. The jugular vein was catheterized to infuse saline, 30% D-glucose, or 30% D-glucose with ML171 (see figure 4-black arrow). Dose dependency was evaluated using 1μM and 5μM doses of ML171. Following, 1 mL Heparin was then administered to preclude blood clotting during intravenous infusion, as well as MABP monitoring via the carotid artery, and blood adherence to the membranes of the free radical sensors inserted into the femoral vein catheters. Hyperglycemic conditions were induced with a loading dose of 30% D-glucose at 0.33mL/min for 3 minutes. Blood glucose levels were maintained around 200mg/dL for 3 hours by measuring blood glucose levels every 20 minutes

using a blood glucose meter and adjusting infusion rate accordingly (0.018-0.025mL/min). To determine NO and H_2O_2 levels in real time throughout the experiment, NO and H_2O_2 microsensors were inserted into catheters in both femoral veins and measured using the free radical analyzer (see figure 5). Levels of NO and H_2O_2 were be measured as pA every 20 minutes until the end of the experiment and converted into concentration (nM for NO and μM for H_2O_2, respectively) using the calibration curves.

Experimental Groups

There were 4 experimental groups:

1. Saline Controls (n=8 for NO and n=9 for H_2O_2) The animals had the same surgical conditions as experimental groups and were monitored for NO and H_2O_2 in a similar manner for 3-hour experimental period following the 1-hour baseline readings. This group was used to demonstrate stability of the physiological parameters under surgery, to demonstrate real-time NO and H_2O_2 levels, and served as a control for the to hyperglycemic group.
2. Hyperglycemic groups (n=8 for NO and n=9 for H_2O_2) These animals were initially infused with 30% glucose to induce hyperglycemia then infused continuously with 30% glucose to maintain hyperglycemic conditions of ≥200 mg/dL throughout the experiment. The groups were used to demonstrate real-time NO and H_2O_2 under hyperglycemic conditions.
3. Hyperglycemic groups + 1 μM ML171 (n=5 for NO and n=8 for H_2O_2) These animals were initially infused with 30% glucose to induce hyperglycemia

then infused continuously with 30% glucose to maintain hyperglycemic conditions of ≥200 mg/dL throughout the experiment. These groups were used to demonstrate real-time NO and H_2O_2 under hyperglycemic conditions with the addition of the NOX1 inhibitor.

4. Hyperglycemic groups + 5 1 μM ML171 (n=5 for NO and n=5 for H_2O_2) These animals were initially infused with 30% glucose to induce hyperglycemia then infused continuously with 30% glucose to maintain hyperglycemic conditions of ≥200 mg/dL throughout the experiment. These groups were used to demonstrate real-time NO and H_2O_2 under hyperglycemic conditions with the addition of a higher dose of the NOX1 inhibitor.

Outline of Experiment

1. Calibrations
2. Animal preparations-
    a. femoral veins: NO and H_2O_2 sensors
    b. Jugular vein: IV infusion with one of experimental group variations
    c. Carotid artery: measure MABP
3. Run experiment-180 minutes
    a. Record: blood glucose, NO and H_2O_2 levels, MABP

Statistical Analysis

All data from text and figures were analyzed by ANOVA using post hoc analysis with the Student- Newman- Keuls post hoc test for pairwise comparison of

groups, $p<0.05$ were considered to be statistically significant. They are represented as mean ± the standard error of the mean (SEM).

Results

Acute Hyperglycemia levels maintained among groups

Blood glucose levels among saline control, 30% D-glucose, 30% D-glucose with 1 μM ML171, and 30% D-glucose with 5 μM ML171 were illustrated in figure 6. The saline control remained in normal blood glucose range (<126mg/dL) for the entire experiment. All three groups treated with 30% D-glucose maintained a blood glucose level over 200 mg/dL for the 180-minute experiment once fully induced. The treatment drug, ML171, showed no altering effects on blood glucose levels, for both the 1 μM ML171, and 5 μM ML171.

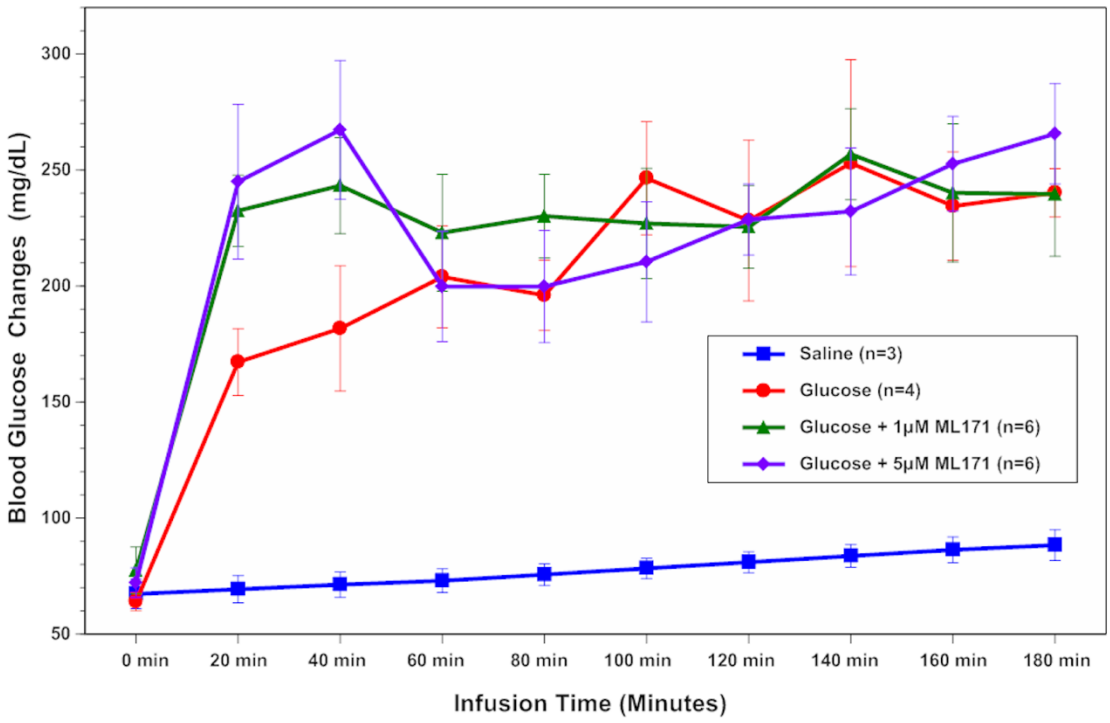

Figure 7. Blood glucose changes throughout 180-minute experiment with 30% D-glucose. Groups infused with 30% D-glucose maintained blood glucose levels of ≥200 mg/dL compared to saline (<126mg/dL).

Mean Arterial Blood Pressures Remained Normal throughout Experiment

Mean arterial blood pressure (MABP) among saline control, 30% D-glucose, 30% D-glucose with 1 μM ML171, and 30% D-glucose with 5 μM ML171 were shown in figure 7. MABP remained in normal range (90-130mmHg) throughout the experiment. Acute hyperglycemia as well as hyperglycemia combined with ML171 had no effect on MABP, which were similar to that of the saline group.

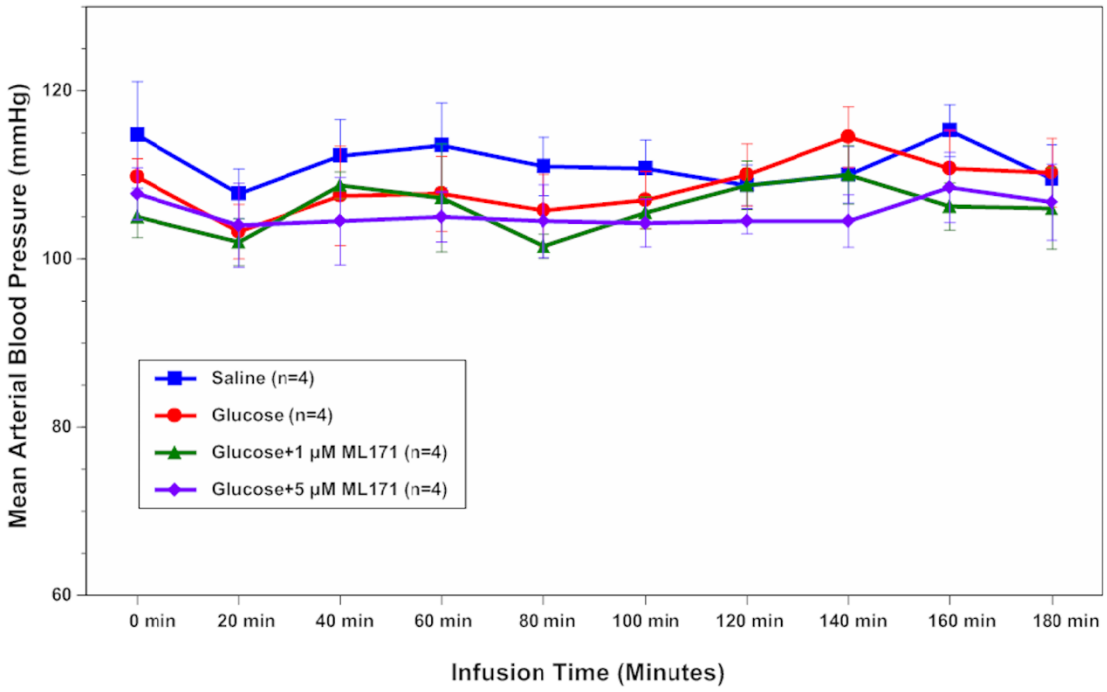

Figure 8. Mean Arterial Blood Pressure among groups taken every 20 minutes for 180-minute experiment. MABP among all groups remained remained in normal range 90-130mmHg for 3 hours.

Acute Hyperglycemia Decreased Blood NO compared to Saline Control

Blood NO levels among saline control and 30% D-glucose were demonstrated in figure 8. The 30% D-glucose group had significant decrease in blood NO levels after 60-minute infusion compared to the saline control. This marked decrease was by a mean value of 37.00±5.12 nM relative to saline ($P<0.05$, $n=8$). The significant reduction in blood NO levels continued and was 101.41±10.91 nM relative to the saline group at the end of the 180-minute infusion ($P<0.01$, $n=8$).

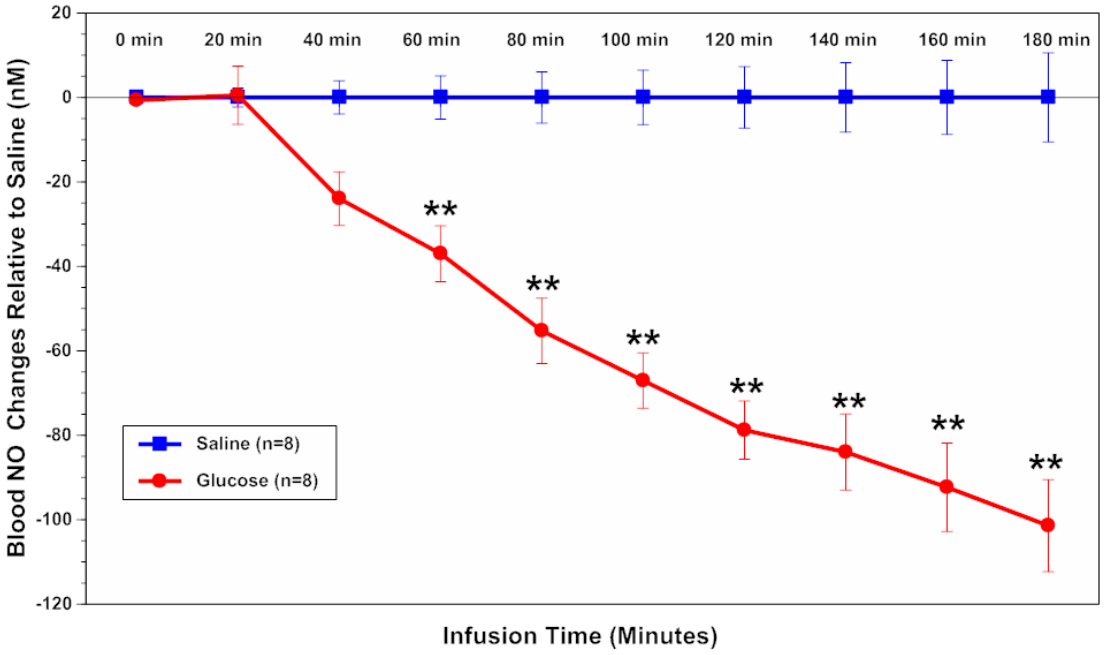

Figure 9. The comparison of blood NO levels relative to saline with 30% D-glucose (*p<0.05, **p<0.01 vs saline). Hyperglycemic group had significantly reduced blood NO levels relative to saline.

Ml171 attenuated decreased blood NO due to acute hyperglycemia in a dose-dependent matter

Blood NO levels among 30% D-glucose, 30% D-glucose with 1 μM ML171, and 30% D-glucose with 5 μM ML171 relative to saline are shown in figure 9. The 30% D-glucose group, as previously stated, had a significant decrease in NO from 60 minutes and throughout the rest of the experiment. The blood NO levels were finally decreased by 101.41±10.91 nM relative to saline ($P<0.01$, $n=8$) . By contrast, the 1 μM ML171 attenuated the decrease in blood NO levels with significant improvement at 100 minutes until the end of experiment. The blood NO levels were only reduced by 32.93±12.67 nM relative to saline at 180 minutes ($P<0.01$, $n=5$). The 5 μM ML171 attenuated the decreased blood NO levels even further with significance seen at 80 minutes into the experiment and continued until completion at 180 minutes with only a slight decrease of 15.46±8.13 nM relative to the saline group ($P<0.01$, $n=5$).

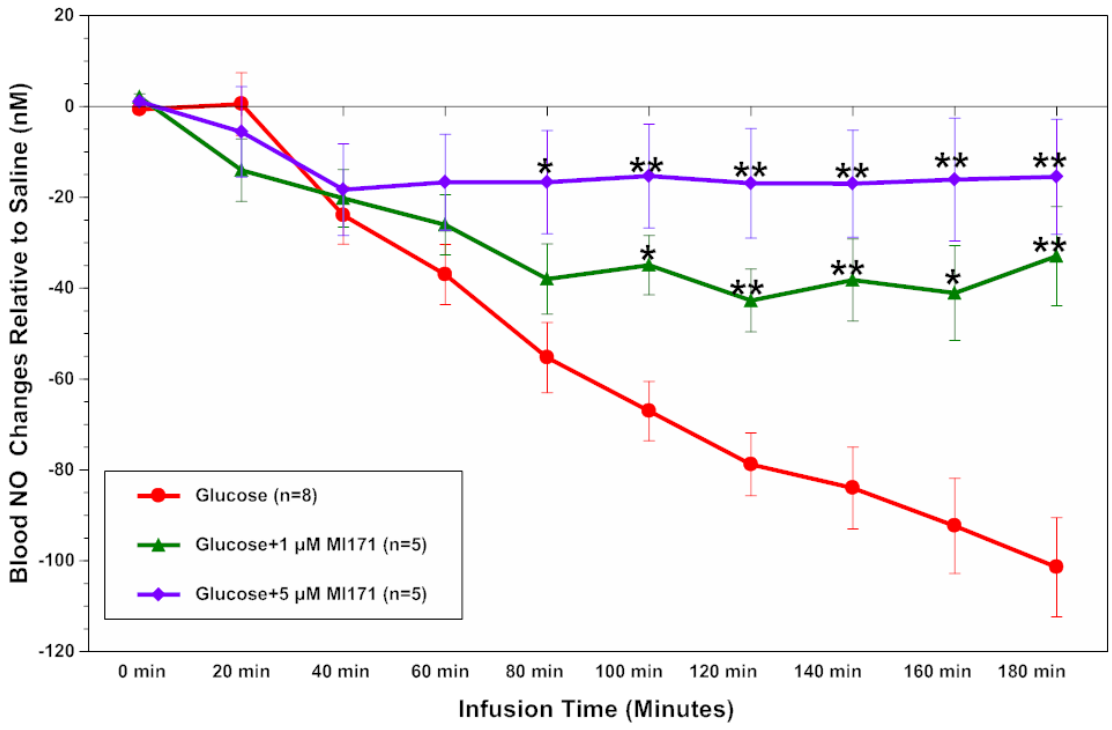

Figure 10. The comparison of blood NO levels relative to saline among 30% D-glucose, 30% D-glucose with 1μM ML171, and 30% D-glucose with 5μM ML171(*p<0.05, **p<0.01 vs Glucose). ML171 increases blood NO levels under hyperglycemia for both 1μM and 5μM ML171 compared to hyperglycemic group.

Acute Hyperglycemia Increased Blood H_2O_2 compared to Saline Control

Blood H_2O_2 levels among saline control and 30% D-glucose are shown figure 10. The 30% D-glucose group had significant increase in blood H_2O_2 levels after 80 minutes with an elevation of 1.37±0.21 μM compared to saline group ($P<0.05$, n=9). The increase in blood H_2O_2 resulted in a final increase at 180- minutes of 3.06±0.42 μM compared to saline ($P<0.01$, n=9).

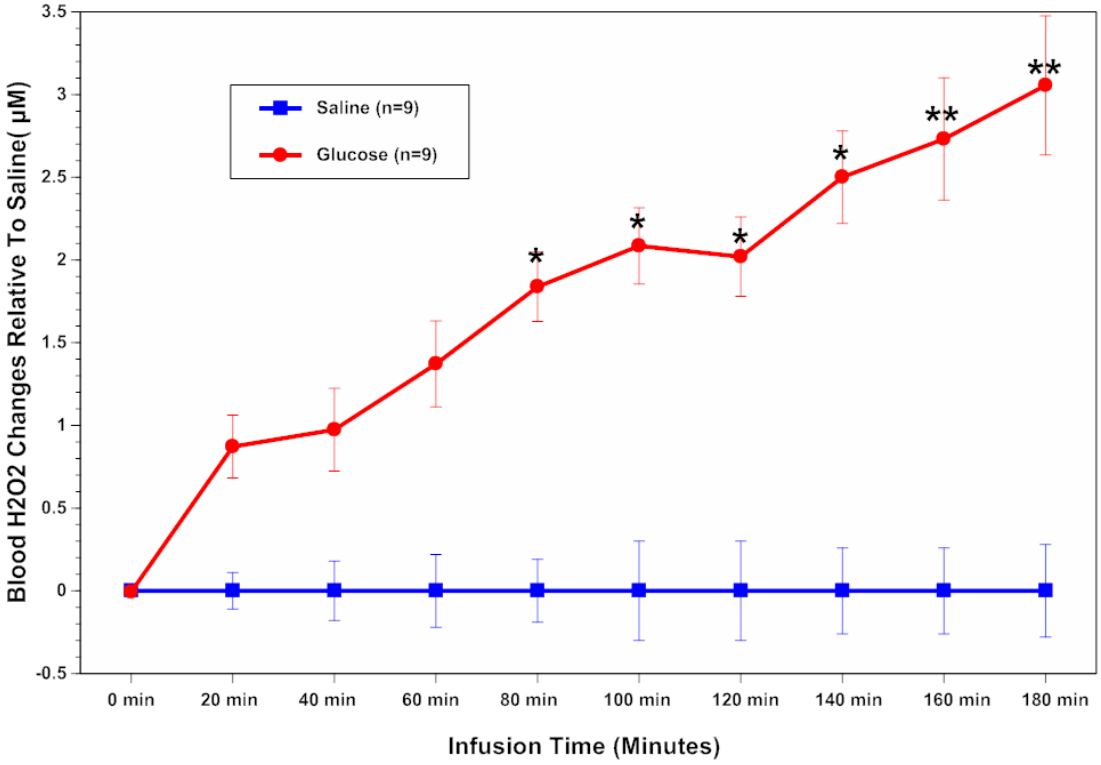

Figure 11. The comparison of blood H_2O_2 levels relative to saline with 30% D-glucose (*p<0.05, **p<0.01 vs saline). Hyperglycemic group had significantly elevated blood H_2O_2 levels relative to saline.

Ml171 attenuated increased blood *H_2O_2 due to Acute Hyperglycemia in dose dependent matter*

Blood H_2O_2 levels among 30% D-glucose, 30% D-glucose with 1 μM ML171, and 30% D-glucose with 5 μM ML171 were demonstrated in figure 11. The 30% D-glucose group, as previously stated, had a significant increase in H_2O_2 starting at 80 minute infusion and continuing until 180 minutes, with a final elevation of 3.06±0.42 μM relative to saline ($P<0.01$, $n=9$). The 1 μM ML171 attenuated the increase in blood H_2O_2 levels with significance at 80 minutes as well as at 140 and 180 minutes

concluding with lower H_2O_2 levels of 1.20±0.61 μM relative to saline (P<0.05, n=8). The 5 μM ML171 attenuated the increased blood H_2O_2 levels even further with significance seen starting at just 40 minutes into the experiment and continued until completion at 180 minutes with blood H_2O_2 levels resulting 1.79±1.02 μM (P<0.01, n=5) lower than the saline control.

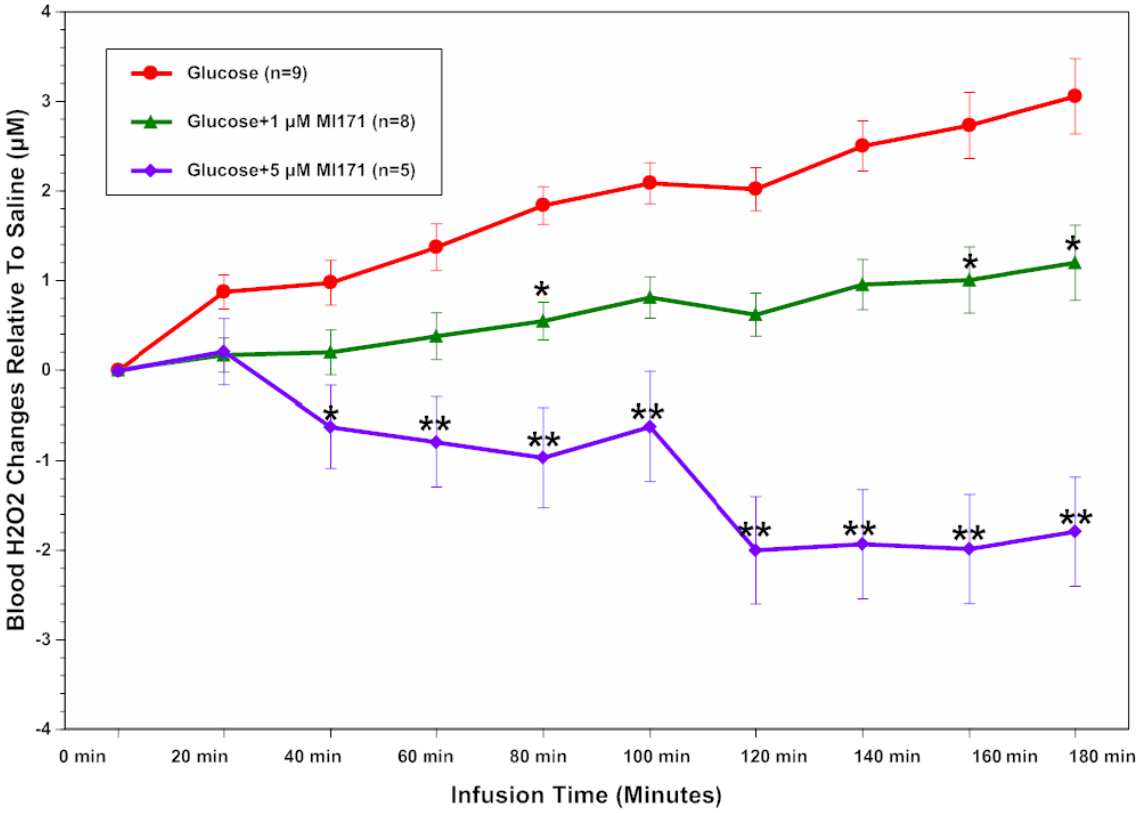

Figure 12. The comparison of blood H_2O_2 levels relative to saline among 30% D-glucose, 30% D-glucose with 1μM ML171, and 30% D-glucose with 5μM ML171(*p<0.05, **p<0.01 vs Glucose). ML171 reduced blood H_2O_2 levels under hyperglycemia for both 1μM and 5μM ML171 compared to hyperglycemic group.

Discussion

These results indicated that during hyperglycemia the blood NO levels were significantly reduced over the 3-hour experiment when compared to saline control. The results also demonstrated that blood H_2O_2 levels were significantly elevated over the 3-hour experiment when compared to saline control.

With the addition of ML171, a selective inhibitor of NOX1, the reduced levels of NO seen in hyperglycemic conditions were attenuated during the 3-hour experiment. ML171 also attenuated the elevation in blood H_2O_2 due to hyperglycemia. The higher dose of ML171 (e.g. 5μM) provided more beneficial effects in restoring blood NO levels and reducing blood H_2O_2 levels compared to the lower dose of ML171 (e.g. 1 μM).

Acute hyperglycemia has been shown to reduce vascular endothelial dependent vasodilation. In 1998, Akbaris et al had determined that endothelium-dependent vasodilation is impaired during acute hyperglycemia in both the micro- and macrocirculation in 20 healthy subjects[27]. The macrocirculation diameter was monitored through the brachial artery by using high-resolution ultra sound before (fasting) and after ingesting 75 g of glucose. The microcirculation diameter was measured by changes in the erythrocyte flux after acetylcholine iontophoresis. Their results showed that dilation was greater during fasting compared to hyperglycemia, suggesting that this impairs dilation. Moreover, animal experiments also suggest that acute hyperglycemia can induce vascular endothelial dysfunction. Gross et al, in 2010 came to such conclusions analyzing shear stress to the left anterior descending coronary arteries(LAD) in dogs. The animals were fasted,

anesthetized, and infused with different doses of glucose, depending on their group. The blood flow, diameter and velocity were measured with response to acetylcholine to measure endothelial function and ROS generation was determined by fluorescence of myocardial biopsies. This study determined that hyperglycemia affects endothelial dysfunction in a dose related manner, as well as ROS production, and decreased shear stress[28]. The induction of vascular endothelial dysfunction may also depend on the time length of acute hyperglycemia and blood vessel. This was seen with McNauty et al in 2011 while measuring the coronary blood flow velocity and diameter in 12 cardiac transplant patients before and after 60-minute glucose infusion. They found that although there were higher levels of oxidative stress, there was no difference in dilated segments or increase blood flow[29]. They suggested that the endothelial dysfunction operates over a longer period of time[29].

This study directly monitors blood NO levels which may serve as a biomarker for vascular endothelial dysfunction in real time under acute hyperglycemia. The current experimental study induced acute hyperglycemia with blood glucose levels of approximately 200- 300 mg/dL for 3 hours. Results determined that blood NO levels were significantly reduced in the hyperglycemia group compared to saline (101.41±10.91 nM; n=8). When considering absolute values from previous studies such as Giustarini et al. in 2004, which found that by measuring nitrites in the plasma of 20 healthy patients it depicted relative levels of NO in the blood[34]. This is assumed because nitrites are found to be the most concentrated form of storage NO carrier[34]. They determined that the average amounts of nitrite in healthy

individuals were measured to be 221±72 nM[34]. Relating back to the current study, NO bioavailability reduction due to hyperglycemia could be very significant.

Many studies indicate that acute hyperglycemia can quickly induce blood oxidative stress and certain molecules can reverse some effects to an extent. Gross et al had also found that the effects of superoxide dismutase mimetic 4-hydroxy-2,2,6,6,-tetramethylpiperidine-1-oxyl, otherwise known as tempol, normalized the oscillatory shear stress that was decreased due to hyperglycemia[28]. Title et al, in 2007 found that Vitamins C and E attenuated the decrease in flow-mediated dilation caused by hyperglycemia[30]. Oxidative stress in some studies is analyzed by lipid peroxidation(LPO). LPO occurs when the free radical attack polyunsaturated FA and further interacts with heavy metals resulting in alkoxyl or peroxl radical that can continue a chain reaction[21]. Iron salts can also react with the LPO, resulting in membrane structure changes[21]. LPO may cause further damage to patients with hyperglycemia and diabetes through oxidation of enzymes, stimulation of proinflammatory cytokines, degenerative changes, and DNA damage[25]. Lipid peroxidation(LPO) is typically measured using Malondialdehyde (MDA) because it is an end product of LPO and is a tag for oxidative stress. Bastos et al, when studying the impact of systemic and local LPO level on periodontal inflammation and disease had found that uncontrolled diabetic patients had 2.5 times higher MDA plasma levels than non-diabetic, and 1.5 times higher for well controlled diabetic patients[25]. LPO was significantly higher in diabetes patients, with a strong correlation ($p<0.0001$). They also discovered that there was positive association between local inflammatory cytokines in the gingival crevicular fluid with diabetic patients. This

also supports previous findings about decreased wound healing due to diabetes and hyperglycemia[25]. The current study measured oxidative stress through a real-time micro sensor for H_2O_2. Real-time H_2O_2 was measured instead of the SO produced since SO is quickly converted to H_2O_2 by superoxide dismutase and is not as stable as H_2O_2. The results determined that blood H_2O_2 levels were significantly elevated in the hyperglycemia group compared to saline. Although the absolute values of H_2O_2 in the blood have been controversial, a recent study by Forman et al. has complied research and analyzed possible true values for H_2O_2[35]. They postulate that the normal range of H_2O_2 in the blood is between 1-5 μM[35]. When considering this with the current study with hyperglycemia increasing blood H_2O_2 by 3.0 ± 0.5 μM, the amount of elevation could be significant and detrimental to physiological conditions.

The application of antioxidants for counteracting the oxidative stress which participates in cardiovascular diseases is questionable due to failure of clinical trials. Vitamin E has been the main vitamin of focus for antioxidant capabilities, but despite promising cohort studies, randomized clinical intervention trials have failed to show any benefit for preventing atherosclerosis or cardiovascular events[33]. There also has been reason to question targeting ROS with antioxidants, because they may only be specific in reducing the concentration of some ROS, but not the others[33]. Therefore, it may be more advantageous to determine the major and initial source for oxidative stress under hyperglycemia.

NADPH oxidases are responsible for the natural production of SO/H_2O_2 in the body. Under physiological conditions this is used to assist the body with innate

immunity, redox-dependent signaling, and production of hormones[33]. In pathological conditions, activation of NADPH oxidases may serve as a key component in mediating hyperglycemia caused oxidative stress and vascular endothelial dysfunction. Taye et al found that by inhibiting NADPH oxidase with apocynin, a specific NAPDH oxidase inhibitor, the elevated levels of their subunit, $p47^{phox}$ were lower as well as the oxidative stress due to hyperglycemia[31]. Activated NADPH oxidase will produce a large amount of SO/H_2O_2. Moreover, it can cross-talk and recruit other possible sources of oxidative stress, such as xanthine oxidase, eNOS uncoupling, and mitochondria, such upregulation of one will activate another[22]. Ago, et al has suggested that xanthine oxidoreductase exists in 2 forms in order to assist in protein catabolism, xanthine dehydrogenase and xanthine oxidase[26]. They state, that in oxidative stress, the oxidoreductase exists primarily in the xanthine oxidase form, producing SO[26]. Current studies have also identified a possible "cross-talk" with mitochondria of NADPH oxidases in the vasculature[22]. The elevation of NADPH oxidase specific SO and H_2O_2 causes mitochondrial dysfunction, leading to the upregulation of SO produced by the mitochondria[22]. This production of SO from the mitochondria in turn further activates the NADPH oxidases. Furthermore, overproduction of ROS from mitochondria can further cause eNOS uncoupling. The eNOS enzyme is then uncoupled, and instead of forming NO to protect homeostasis and regulation, eNOS quenches SO and peroxinitrite (another free radical) is formed. Thus, the futile cycle resulting in endothelial dysfunction is created. The damage associated with elevated ROS is in part due the eNOS uncoupling by the SO. The free radical SO quenches NO and forms

peroxinitite. This is an even more toxic free radical, due to its ability to uncouple eNOS. This is accomplished by oxidizing the complex as well as its cofactor BH_4 [18].

NADPH oxidases are expressed throughout the body. NOX1, 2 and 4 are expressed specifically in the vasculature, with 1 and 2 present in endothelial cells. NOX1 is also highly expressed in VSMCs[26]. Bertolet et al has shown that NOX2 inhibition with gp91ds-tat has only shown partial attenuation of H_2O_2 under hyperglycemia[7]. The role of NOX1 in acute hyperglycemia is unclear. Weaver et al, research had found that NOX1 inhibition can preserve the function of pancreatic beta cells through the use of ML171[11]. We found that ML171 can attenuate the oxidative stressed caused by the upregulated NOX1 during hyperglycemia.

NOX1 inhibition can reduce PKC activation, which is the upstream signaling of NADPH oxidase activation. In diabetic kidney, NOX1 has been suggested to be linked to activation of PKC activation[8]. This is the first proposed feed forward mechanism of NOX1 with PKC. It was also suggested that NOX1 but not NOX2 mRNA expression was elevated in early hyperglycemia[8]. NOX1 deficient mice had reduced oxidative stress and reduced activation of PKC. NOX1 in hyperglycemia induced oxidative stress and vascular endothelial dysfunction was focus of our hypothesis.

It was suggested by Youn, et al that NOX1 mediates BMP4-induced eNOS uncoupling, shown by a diminished BMP4-induced eNOS uncoupling with NOX1 siRNA-transfected cells[17]. This was demonstrated by eNOS uncoupling present within endothelial cells when exposed to BMP4 or bone morphological protein 4[17]. NO is responsible for vasodilation, inhibiting platelet, and white blood cell

activation. These actions are necessary for maintaining vascular homeostasis. The product shift of uncoupled eNOS from NO to SO not only diminishes NO bioavailability that maintains homeostasis, but also increases SO products, which results in more oxidative stress. The possible mechanism of what may occur in the instance of blocking NOX1 with its involvement with other enzymes is outlined in figure 13. The current study found that ML171, a selective NOX1 inhibitor, significantly reduced acute hyperglycemia-induced H_2O_2 increase and NO decrease. The higher dose provided better effects than lower dose.

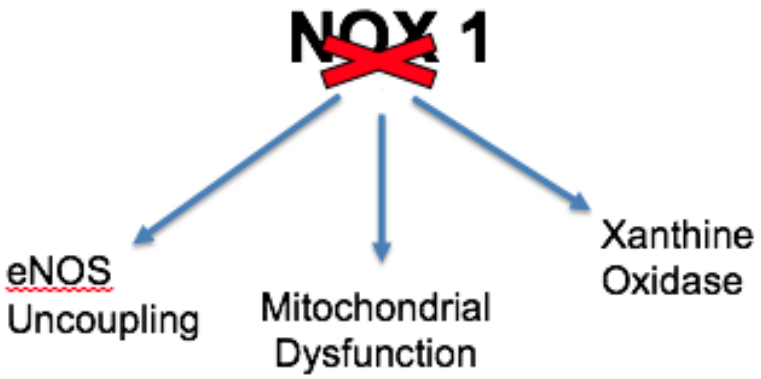

Figure 13. Possible mechanism of NOX1 activity at the endothelial level. NOX1 may influence other enzymes and their production of SO.

To further understand the role of NOX1 in hyperglycemia-induced vascular dysfunction and oxidative stress, future studies will be designed to measure blood nitrite and other oxidative stress indicators such as Malondialdehyde (MDA). The impact of body temperature on blood NO and H_2O_2 levels also were measured, NO levels slightly decreased in the saline control group and there was a slight increase in H_2O_2. Future studies could employ westernblot and immunohistochemistry to

understand the mechanism as well as interaction of NOX1 with mitochondrial ROS, uncoupled eNOS, and PKC under the present experimental conditions.

Future studies include clarifying the effects of body temperature, to rule out any variables due to heat loss during experiment. A heating pad will be placed under the animals for the duration of the study. The long term effects of ML171 has also not yet been explored, and this could be an additional aspect of future studies.

The results of this study must be considered in light of certain limitations; for the absolute values could not be measured. The lab has not yet confirmed the blood plasma for H_2O_2 and NO, but a method to do so will be incorporated into future studies. The free radical microsensors used in this study are also sensitive to movement and other variables, which may influence real-time readings. Future studies may incorporate other ways to measure NO and H_2O_2 in real-time by use of fluorescence kits if a protocol is developed successfully for this experimental design.

Summary

Acute hyperglycemia is harmful, and the futile cycle created by NOX1 and uncoupling of eNOS is responsible for vascular endothelial damage. Focus on reduction of oxidative stress by inhibiting NOX1 may prevent the vicious cycle of causing oxidative stress and NO reduction in the blood due to hyperglycemia.

Acknowledgments

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