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The Real-Time Measurements of Blood Nitric Oxide (NO) and Hydrogen Peroxide (H2O2) Levels under Acute Hyperglycemia

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The Real-Time Measurements of Blood Nitric Oxide (NO) and Hydrogen Peroxide (H_2O_2) Levels under Acute Hyperglycemia

A Thesis in Vascular Endothelial Dysfunction by Michael Minni

Submitted in Partial Fulfillment of the Requirements for the Degree of Masters in Biomedical Sciences
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This thesis has been presented to and accepted by the Associate Dean for Curriculum and Research Office of Philadelphia College of Osteopathic Medicine in partial fulfillment of the requirements for the degree of Master of Science in Biomedical Sciences.

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.

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Abstract

Vascular endothelial dysfunction is one of the earliest recognizable events under hyperglycemic conditions. It is characterized by decreased endothelium-derived nitric oxide (NO) bioavailability and increased oxidative stress, such as superoxide and hydrogen peroxide (H_2O_2) overproduction. However, the real-time changes in blood NO and H_2O_2 levels under acute hyperglycemia have not been evaluated. In this study, acute hyperglycemia (200 mg/dl, 400 mg/dL, and 600 mg/dL) was induced by intravenous infusion of 20%, 30%, and 50% D-glucose respectively for 180 min. Infusion of saline or 30% L-glucose serve as controls. Blood NO or H_2O_2 levels were measured at real-time by inserting calibrated NO or H_2O_2 microsensors (100 μm diameter) into each femoral vein, respectively. In the saline group, blood NO levels remained stable and only slightly decreased by 17.61±8.04 nM (n=7) at 180 min compared to baseline. By contrast, hyperglycemia significantly decreased blood NO levels from 100-160 min to the end of the experiment. At 180 min, blood NO levels in 200 mg/dL, 400 mg/dL, and ≥600 mg/dL groups were 71.3±17.9 nM (n=7), 112.15±15.28 nM (n=6), and 105.98±23.45 nM (n=6) lower than that in saline group, respectively (all $p<0.01$). However, there was no significant difference in blood NO levels between L-glucose group and the saline control throughout the entire experiment. Blood NO levels were only 11.58±22.01 nM (n=5) higher compared to the saline control at 180 min of infusion of 30% L-glucose. Meanwhile, blood H_2O_2 levels in saline group continued to drop and reduced by 4.62±0.34 μM (n=5) at the end of the 180 min infusion compared to the baseline. By contrast, hyperglycemia significantly increased blood H_2O_2 levels from 20-60 min, then stayed higher throughout the rest of the experiment. At 180 min, blood H_2O_2 levels in 200 mg/dL, 400 mg/dL, and 600 mg/dL groups were 2.40±0.61 μM (n=6), 3.1±0.2 μM

(n=6), and 2.21±0.37 μM (n=6) higher than that in saline group, respectively (all $p<0.01$). However, blood H_2O_2 levels in L-glucose group remained similar level as saline control throughout the experiment. The blood H_2O_2 levels were only 0.88±0.29 μM higher in the L-glucose group (n=5) compared to the saline control at the end of the experiment. In summary, this acute hyperglycemia rat model exhibits vascular endothelial dysfunction by presenting significantly lower blood NO levels and significantly higher blood H_2O_2 levels, which is not principally due to hyperosmolarity.

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Acknowledgments

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Introduction

Hyperglycemia and Vascular Complications

The conditions of hyperglycemia has been emphasized in many diseases, such as diabetes, metabolic syndromes, and some post-surgery complications (Fatehi-Hassanabad, Chan et al.; Ceriello, Hanefeld et al. 2004; Ceriello 2005; Forstermann 2010). There is considerable evidence indicating that both acute and chronic hyperglycemia have deleterious effects on vascular function and are closely related to micro and macro vascular complications. However, the mechanisms underlying hyperglycemia-induced vascular and organ damage are complex and unclear. Therefore, it is critical to understand mechanisms involving vascular endothelial dysfunction under hyperglycemic conditions for identifying and ultimately developing new treatment strategies to attenuate the detrimental effects of hyperglycemia.

1. Clinical Hyperglycemia Conditions

In normal human subjects, the concentration of fasting whole blood glucose is around 101 mg/dL. Hyperglycemia occurs when an individual's blood glucose level exceeds 126 mg/dL. If hyperglycemia persists after at least an 8 hr overnight fast, a diagnosis of diabetes is made. Fasting blood glucose within 110 to 124 mg/dL is recognized as impaired fasting glucose (Gardner and Shoback 2007). The oral glucose tolerance test is another way to diagnose diabetes. This test requires an individual to receive 75 g of glucose solution and then wait for 2 hours, then the blood glucose levels will be checked. If an individual's blood glucose levels exceed 180 mg/dL, then a

diagnosis of diabetes is made. Two-hour postprandial blood glucose levels between 121-178 mg/dL are considered as impaired glucose tolerance. When the blood glucose is about 200 mg/dL (i.e. mild hyperglycemia), patients begin to exhibit the symptoms of hyperglycemia including excessive thirst, polydipsia, headaches, fatigue and blurred vision (Forstermann and Munzel 2006; Gardner and Shoback 2007; Forstermann 2010). Ketoacidosis is a complication of diabetes that occurs when the body cannot use glucose as a fuel source due to insufficient amount of insulin in the body, and fat is utilized instead. This affects individuals with a blood sugar level above 600 mg/dL. This severe hyperglycemia can cause weakness and may progress to coma or even death. When blood glucose is 700 mg/dL or higher, it can induce hyperglycemic hyperosmolar syndrome with more viscous blood due to the excess glucose in the blood and may result in brain damage (Ceriello 2005).

2. Acute Hyperglycemia in Non-Diabetic Subjects

In non-diabetic subjects, blood glucose levels may increase under some conditions such as after a high glucose diet (i.e., oral glucose test), stress, post-surgery, medicine, and critical illness (Finney, Zekveld et al. 2003; Falciglia, Freyberg et al. 2009; Sato, Carvalho et al. 2010). Clinically, fasting plasma glucose and postprandial (2 hours after meal) glucose levels or oral glucose tolerance test often have been used to indicate the acute hyperglycemia level.

Both critical illness and postoperative status are accompanied with so-called stress-induced hyperglycemia. It is a transient hyperglycemia during illness rather than from previous diabetes mellitus (Dungan, Braithwaite et al. 2009). Evidence indicates

that stress-induced hyperglycemia in critically ill and preoperative patients serves as an independent marker of poor outcomes, such as infection, slow recovery, and higher mortality (Finney, Zekveld et al. 2003; Falciglia, Freyberg et al. 2009; Sato, Carvalho et al. 2010). If hyperglycemia is left uncontrolled, it can lead to hypokalaemia, hypoatraemia, arrhythmias and an increased risk of ischemic brain injury (Hogue, 2006). Also hyperglycemia may predispose patients to an increased risk of post-surgical infections through impaired phagocytic activity and decreased neutrophil function (Rassias, Marrin et al. 1999; Hanazaki, Maeda et al. 2009). Due to the possible danger caused from hyperglycemia, it is important to make sure that patients maintain a normal blood glucose at all times. Published evidence suggests blood glucose less than 110mg/dL in critically ill, surgical and non-surgical patients, reduces morbidity and mortality (van den Berghe, Wouters et al. 2001).

Some studies have linked the role of acute hyperglycemia to the development of vascular complications in non-diabetic individuals (Forstermann and Munzel 2006). It suggests that even in the absence of clinically diagnosed diabetes, high dietary glucose consumption and postprandial hyperglycemia are associated with an increased risk of cardiovascular disease (Ceriello, Hanefeld et al. 2004). Hanefeld et al. have linked postprandial hyperglycemia levels in nondiabetic patients with the incidence of mediointimal carotid thickening. In a study containing 403 participants, Hanefeld noted a correlation between significant intima-media thickness in the carotid arteries of non diabetic individuals and within the 2 hour postprandial glucose spiking (Hanefeld, Koehler et al. 1999). Further evidence suggest that postprandial glucose levels are associated with increased production and disturbed removal of triglyceride-rich lipoporteins, impaired fibrinolysis and oxidative stress. Hanefeld et al. suggested that

postprandial hyperglycemia with oxidative stress may have damaging effects on the arterial wall and could accelerate atherosclerosis. It also has been suggested that most cardiovascular risk factors may be modified in the postprandial phase and are directly affected by an acute increase in blood glucose. One of the primary mechanisms is that acute hyperglycemia may cause the overproduction of free radicals, which favors the development of vascular endothelial dysfunction (Ding, Aljofan et al. 2007).

3. Chronic Hyperglycemia/Diabetes

Diabetes mellitus is one of the most common chronic diseases worldwide and is associated with both increased morbidity and mortality. It is also characterized by chronic hyperglycemia resulting from defects in insulin sensitivity and/or secretion associated with different vascular complications (ADA, Diabetes Care 2007). Currently, there are about 285 million individuals with diabetes worldwide and by the year 2030, this prevalence is expected to increase up to 439 million globally (Wild, Roglic et al. 2004). Long term prognosis of individuals with diabetes remains poor because of the microvascular and macrovascular complications resulting from chronic hyperglycemia.

Diabetic microvascular complications include retinopathy, nephropathy, and neuropathy. In 2009, the American Diabetes Association published data showing diabetes is the leading cause of new cases of blindness and kidney failure among adults aged 20–74 years. Moreover, about 60% to 70% of people with diabetes have mild to severe forms of nervous system damage. Furthermore, greater than 60% of nontraumatic lower-limb amputations occur in people with diabetes (ADA, 2009). In regard to macrovascular complications, diabetes is characterized by high incidence of cardiovascular diseases (Ceriello 2005). Type II diabetics are two to four times more

likely to develop cardiovascular diseases than non diabetic individuals. Sixty-five percent of patients with type II diabetes die from complications related to heart disease or stroke (Rodriguez, Lau et al. 1999; Milicevic, Raz et al. 2008).

Postprandial hyperglycemia can play a key role in the pathogenesis of vascular complications in diabetes. The clinical follow up study conducted by Meigs revealed that fasting hyperglycemia and 2-h post-challenge hyperglycemia independently increase the risk for cardiovascular disease, such as heart disease and stroke in diabetic patients (Meigs, Nathan et al. 2002). Furthermore, in the Diabetes Epidemiology: Collaborative analysis of Diagnostic criteria in Europe (DECODE) study, mortality risk of cardiovascular disease increased with post-glucose load plasma glucose concentrations regardless of fasting plasma glucose levels (DECODE Study Group 2001). Postprandial hyperglycemia in type II diabetic patients has also been associated with myocardial perfusion defects, due to the deterioration in heart microvascular function (Scognamiglio, Negut et al. 2006). Similarly, other studies with type I diabetes patients demonstrated that acute hyperglycemia altered myocardial repolarization and increased the stiffness of intermediate-sized arteries (Gordon, 2007).

Hyperglycemia and Vascular Endothelial Dysfunction:

It is very important to maintain the homeostasis of vascular endothelium at resting state for proper tissue/organ blood perfusion. Vascular endothelial can be cultivated by various stimuli and is involved in the pathogenesis of different diseases, such as ischemia/reperfusion injury, hyperglycemia, diabetes, and infectious diseases (Saha, Xia et al. 2006; Pate, Damarla et al. 2010). Hyperglycemia can disturb the homeostasis of

vascular endothelium, induce vascular endothelial dysfunction, and initiate the cascade of vascular-organ dysfunction.

1. Normal Vascular Endothelial Function

The vascular endothelium is composed of a monolayer of endothelial cells. These cells line the vascular system and serve as a semi-permeable barrier separating circulating blood from the vessel wall and control the transfer of molecules between intravascular and extravascular spaces (Sumpio, Riley et al. 2002). Normally, the vascular endothelium stays at resting state and acts as an important regulator of vascular homeostasis (Watson, Goon et al. 2008). Endothelial cells can also detect alterations in hemodynamic forces and respond by synthesizing or releasing a myriad of vasoactive substances including prostacyclin and nitric oxide (Sumpio, Riley et al. 2002; Girn, Ahilathirunayagam et al. 2007; Khazaei, Moien-Afshari et al. 2008). Therefore, under normal physiological conditions, the vascular endothelium primarily serves to provide an antithrombotic surface which facilitates adequate blood flow by regulating thrombosis, thrombolysis, leukocyte adherence, platelet adherence, and vascular tone (Sumpio, Riley et al. 2002; Girn, Ahilathirunayagam et al. 2007; Scalia 2007).

Nitric Oxide

Nitric Oxide (NO), a potent vasodilator, and is the most important vasoactive substance produced by the vascular endothelium (Davignon and Ganz 2004). It serves as a vasodilator to all blood vessels in the body as well as reduces inflammation. Under normal physiological conditions, endothelial derived NO is produced by the membrane bound enzyme endothelial nitric oxide synthase (eNOS) which oxidizes the amino acid,

L-arginine, to form the intermediate N^G-hydroxy-L-arginine (Govers and Rabelink 2001). The two essential cofactors in this reaction are Nicotinamide-adenine-dinucleotide phosphate (NADPH) and tetrahydrobiopterin (BH_4) (Vanhoutte 2003; Channon 2004). The oxygenase domain of eNOS contains a BH_4 prosthetic group. By donating a single electron, BH_4 activates heme bound O_2. This is then recaptured to enable NO release (Verhaar, Westerweel et al. 2004). It has been indicated that L-arginine and BH_4 are critical for stabilizing the dimeration of eNOS structure for electron transfer and NO production. Thus, eNOS normally produces NO in the presence of an essential cofactor, BH_4, by facilitating the reduction of molecular oxygen to L-arginine oxidation and generation of L-citrulline. This reaction is referred to as eNOS coupling (See Figure 1) (Schmidt and Alp 2007).

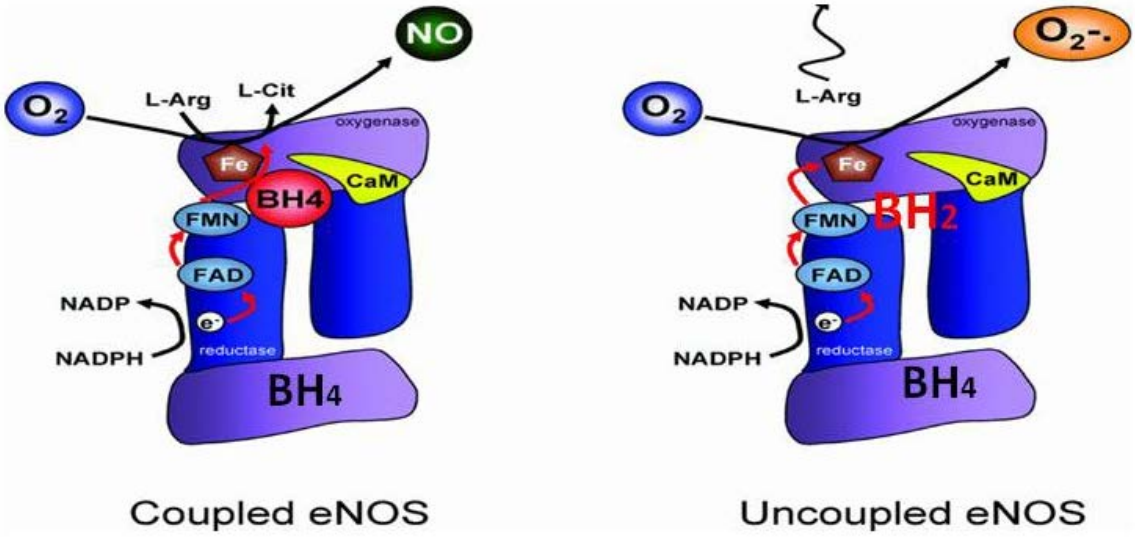


Figure 1. The role of BH_4 in regulating eNOS activity. Endothelial NOS coupling occurs during a normal setting allowing the reaction to produce NO and L-citrulline from L-arginine /NADPH/O_2. When uncoupled, eNOS will produce SO. Picture is adapted from (Schmidt and Alp 2007).

The synthesis and release of NO occur in response to stimuli acting on the endothelial cell surface and are dependent on the intracellular calcium level (Kharbanda and Deanfield 2001). Acetylcholine and physiological stress, such as blood flow shear stress, can stimulate the release of NO (Vanhoutte 2003). This is accomplished by increasing the intracellular calcium level and facilitating the production of NO from eNOS. NO, a potent and lipophilic gas, is able to permeate cell membranes easily and diffuse toward smooth muscle cells and into the vascular lumen (See figure 2) (Watson, Goon et al. 2008).

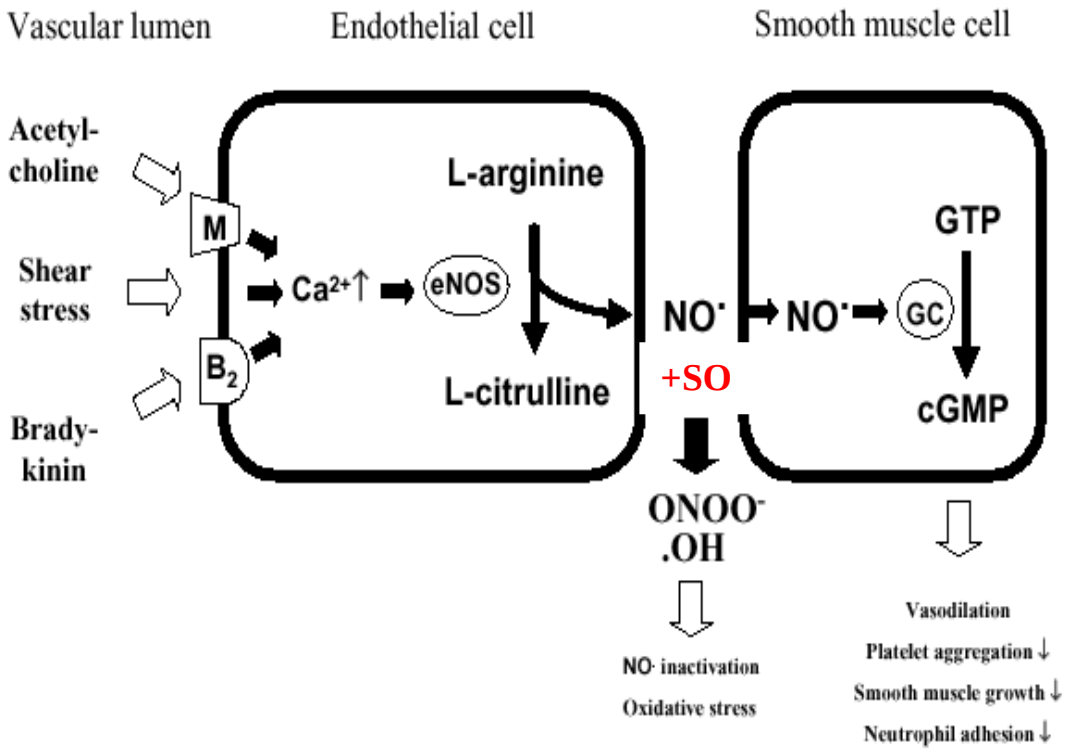


Figure 2. NO production and its physiological and pathophysiological effects.

NO exerts its effect in smooth muscle cells by binding to the heme group of the enzyme guanylyl cyclase (Al-Sa'doni and Ferro 2000). Activated guanylyl cyclase results

in an increased production of cyclic 3’5’ guanosine monophosphate (cGMP). cGMP is an important second messenger which results in dephosphorylation of myosin light chain kinase (MLCK) and the activation of cGMP-dependent protein kinase. GMP-dependent kinase phosphorylates K^+ channels leading to the membrane hyperpolarization and reduction of intracellular Ca^{2+} ions. These collaborative processes result in the relaxation of smooth muscle cells (See figure 2) (Lincoln, Komalavilas et al. 1994; Kharbanda and Deanfield 2001).

In addition to its vasodilatory ability, NO has several effects on the cardiovascular system which include quenching superoxide radicals for its removal from the body as well as its inhibitory effects on smooth muscle cell proliferation (Laroia, Ganti et al. 2003). NO plays an important role in preventing neutrophil adhesion, aggregation, and activation (See figure 2) (Girn, Ahilathirunayagam et al. 2007). Kubes et al. demonstrated that the blockade of NO production by using N^G nitro L-arginine methyl ester (L-NAME), a non-selective NO synthase inhibitor, resulted in a 15-fold increase in leukocyte adherence and significant increase in transmigration of leukocytes in cat mesenteric post-capillary venules (Kubes, Suzuki et al. 1991). These transmigrated leukocytes can ultimately release SO, myeloperoxidase, elastase and cause cell and tissue damage (Lefer, 1996). These results indicate endothelial-derived NO is an important endogenous inhibitor of leukocyte-endothelial interactions (Kubes, Suzuki et al. 1991).

In addition to eNOS, two other isoforms of NOS enzyme exist; and each has specific functions. Neuronal NOS (nNOS) is found in the nervous system and skeletal muscle. NO release from nNOS is used for transmission from one neuron to another while inducible NOS (iNOS) is found in the immune system and utilized by

macrophages, neutrophils, and other inflammatory cells (Forstermann and Munzel 2006; Keklikoglu 2008). Neuronal NOS and eNOS are constitutively expressed and tightly regulated by calcium and calmodulin. The both NOS forms produce small amounts of NO (i.e., nM range), which has precise actions on adjacent cells. In contrast, iNOS is calcium and calmodulin independent and produces high levels of nitric oxide (i.e., μM range) in response to inflammatory cytokines (Mungrue, Husain et al. 2002).

A small amount of NO diffuses into smooth muscle, but the majority of the NO that does not diffuse abluminally and reacts rapidly with hemoglobin to form nitrate. Another fraction of NO produced by eNOS in the vasculature (approximately 20%) escapes inactivation by hemoglobin and is oxidized to nitrite (NO_2^-) in the plasma by ceruloplasmin, and functions to modify proteins and lipids to form low concentrations of *N*-nitrosamines, *S*-nitrosothiols (RSNO), and nitrated lipids. The formation of nitrite may serve as stable storage forms of NO that may enzymatically reduced to NO by deoxygenated hemoglobin along the physiological oxygen and pH gradient (MacArthur, Shiva et al. 2007).

Reactive Oxygen Species (ROS)

ROS are molecules derived from oxygen including superoxide (SO), hydrogen peroxide, and the hydroxyl radical. They are generated from NADPH oxidase, xanthine oxidase, uncoupled eNOS, arachidonic acid metabolizing enzymes (i.e. cytochrome P-450 enzymes), lipoxygenase and cyclooxygenase, and the mitochondrial respiratory chain (Griendling 2005). Normally, they exist at low concentrations and involve physiological roles in host defense mechanism (against infectious agents) and in a number of cellular

signaling systems (Fatehi-Hassanabad, Chan et al.). Moreover, an intricate balance exists between the formation of these oxidizing substances and their removal by antioxidant mechanisms under physiological conditions (Girn, Ahilathirunayagam et al. 2007). For example, superoxide is a very unstable compound and has a half-life of only a matter of seconds. SO is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) and subsequently converted to water by catalase to facilitate its removal from the body. Thus, the measurement of H_2O_2 will serve as a good indicator of blood SO production (Chen, Kim et al. 2010).

2. Endothelial Dysfunction and Hyperglycemia

Vascular endothelium has been considered as an essential component in vascular/organ damage because its dysfunction is a common and early feature (Matsuda and Hattori 2007; Schafer and Bauersachs 2008). Medical literature mostly defines endothelial dysfunction as the impairment of endothelium-dependent vasorelaxation caused by the loss of NO bioactivity in vessel walls (Cai and Harrison 2000), and has been associated with increased oxidative stress (Sharma and Singh 2001). Clinically, noninvasive assessment of endothelial function is to utilize the ultrasound to measure blood vessel diameter and blood flow in order to evaluate vasodilators or flow-mediated vascular endothelial-dependent vasodilation (Ceriello 2005).

Hyperglycemia is a cause of vascular endothelial dysfunction by reducing blood NO bioavailability (Crabtree, Smith et al. 2008). An abundance of accumulated evidence suggests that vascular endothelial dysfunction is one of earliest events induced by hyperglycemia. The oral glucose tolerance test on healthy subjects, which simulates the

postprandial hyperglycemia, results in a rapid reduction in endothelial-dependent vasodilation at 1 hour and is quickly restored to the normal at 2 hour postchallenge. Similarly, the postprandial hyperglycemia in relatively healthy subjects can acutely affect endothelium-dependent regulation of blood flow (Triggle 2007). Furthermore, application of BH_4 to these healthy subjects reverses postprandial hyperglycemia-induced vascular endothelial dysfunction at 1 hour (Triggle 2007). By contrast, the recovery of forearm vascular endothelial function postchallenge is greatly slowed in diabetic patients who have chronic hyperglycemia (Kawano, Motoyama et al. 1999).

Endothelial NOS and Hyperglycemia

Endothelial NOS coupling requires the presence of L-arginine and BH_4. However, when L-arginine is not available or BH_4 is oxidized to dihydrobiopterin (BH_2) which reduces the ratio between BH_4 to BH_2, eNOS becomes uncoupled and produces SO instead of NO by utilizing molecular oxygen (See Figure 1) (Crabtree, Smith et al. 2008). Although it is suggested that eNOS uncoupling can mediate the vascular endothelial dysfunction under hyperglycemia conditions, it is still uncertain that eNOS uncoupling is due to the insufficiency of L-arginine or reduced ratio of BH_4 to BH_2.

Oxidative Stress under hyperglycemia

Oxidative stress refers the process of cellular damage as a result of the uncontrolled action of ROS (Watson, Goon et al. 2008). Oxidative stress occurs in response to disruptions in internal homeostasis and disrupts this balance in favor of ROS production which overcomes the body's defense mechanisms (i.e. SOD) (Girn, Ahilathirunayagam et al. 2007). The pathologies caused by SO are detrimental and well

known (Fan, Sun et al. 2002). SO is known to induce cellular apoptosis and necrosis and impairs vasodilatory responses of the vascular endothelium (Fan, Sun et al. 2002). When present in high concentrations, superoxide can oxidize lipids (lipid peroxidation), proteins, and damage DNA. Under conditions of oxidative stress SO can combine with NO. This will inactivate the vasodilatory effects of NO. This process occurs at a rapid rate of 6.7×10^{9} $mol/L^{-1}\text{-}s^{-1}$ (Thomson, Trujillo et al. 1995). The new compound produced is called peroxynitrite which is very cytotoxic and induces cell damage by further uncoupling eNOS dimers to further produce more SO (See Figure 1). Thereafter, endothelial dysfunction can be induced and also serves as a feedback inhibition of NO production. (Girn, Ahilathirunayagam et al. 2007).

When endothelial cells are exposed to high glucose, SO release significantly increases (Oak and Cai 2007; Forstermann 2010). Furthermore, many studies show that there exists systemic oxidative stress and lipid oxidation under acute and chronic hyperglycemic conditions (McNulty, Tulli et al. 2007). In addition to uncoupled eNOS, another important source of SO production in vascular tissue is though the enzyme NADPH oxidase. This enzyme is activated by high glucose via a PKC- beta dependent process. The use of PKC-beta inhibitors have been shown to prevent hyperglycemia induced endothelium dysfunction (Beckman, Goldfine et al. 2002). Additionally, xanthine oxidase, NADPH oxidase of neutrophils, and mitochondrial dysfunction also are sources for the production of SO. Therefore, anti-oxidants, such as vitamin C helps preserve the vascular endothelial function under hyperglycemia conditions (Beckman, Goldfine et al. 2001).

Vascular endothelial dysfunction can further initiate leukocyte-endothelial interactions. Vascular endothelial adhesion molecules such as P-selectin and intercellular

adhesion molecule-1(ICAM-1) are upregulated to promote leukocyte rolling, adherence and transmigration. Furthermore, transmigrated leukocytes cause inflammatory reactions by releasing proteases, SO, and cytokines such as tumor necrosis factor-α. This inflammation can induce organ damage (Piwkowska, Rogacka et al.; Ceriello, Falleti et al. 1998).

Hypothesis

It is accepted that chronic hyperglycemia induces oxidative stress and reduces endothelial-derived NO bioavailability, which are closely related to microvascular and macrovascular complications of diabetes. There is increasing evidence suggesting that acute hyperglycemia, especially postprandial hyperglycemia, may increase oxidative stress and cause vascular endothelial dysfunction in normal subjects (Inoguchi, Battan et al. 1992; Ceriello, Hanefeld et al. 2004). However, it is still uncertain how quickly the acute hyperglycemia can impair vascular endothelium dependent vasodilation in healthy individuals. Some studies demonstrated that hyperglycemia from one to six hours reduces endothelium dependent forearm circulation of healthy subjects (Williams, Goldfine et al. 1998; Title, Cummings et al. 2000; Beckman, Goldfine et al. 2001). By contrast, according to McNulty, a single episode of acute hyperglycemia for one hour causes systemic oxidative stress but does not affect endothelium-dependent vasodilation on coronary circulation (McNulty, Tulli et al. 2007). In these studies, they use acetylcholine or other vasodilators to evaluate vascular dilatory function but do not directly measure blood NO change in real-time.

In order to further understand the influence of hyperglycemia on vascular function of a normal subject, it is critical to monitor blood NO and oxidative stress (i.e. H_2O_2) in real-time under hyperglycemic conditions. Our lab has established a novel method for the real-time measurement of blood NO and H_2O_2 from rat femoral veins (Teng, Kay et al. 2008; Chen, Kim et al. 2010). This study aims to establish an acute hyperglycemic animal model to evaluate the change of blood NO and H_2O_2 concentrations in real time.

We hypothesize that in the saline control, NO release may be slightly reduced due to the decreased body temperature. There will be a decrease in H_2O_2 release. In contrast, hyperglycemia will induce greater decrease in NO compared to saline control. Furthermore, H_2O_2 release will remain at a higher level in the hyperglycemic group as opposed to the saline group.

Methods

NO or H_2O_2 Microsensor Calibration

Before performing the animal preparation, the NO or H_2O_2 microsensors (100 μm, World Precision Instruments (WPI), Sarasota, FL) were calibrated as per manufacturer's recommendations. The specificity of the free radical microsensors is dependent on the selective membrane covering the sensor. When NO or H_2O_2 in the solution or biological fluid diffuse through the membrane, it is oxidized and an electrical (oxidation/reduction) signal is generated of which amplitude is proportional to the free radical concentration in the sample. Each type of sensor has a selective poise voltage important for obtaining reliable data, with high performance Faraday shield incorporated in the sensor to minimize environmental noise (Zhang, Ju et al. 2008).

The sensor is calibrated by constructing a standard curve using known concentrations of the free radical of interest, enabling conversion of the electrical signal recorded during the experiment to a molar concentration of NO or H_2O_2. The NO and H_2O_2 microsensors soaked in 10 mL copper sulfate and 10 mL of PBS respectively to retrieve a baseline. Moreover, the microsensors were connected with cables which were plugged into the Apollo 4000 free radical analyzer (WPI, Sarasota, FL). For calibrating NO microsensor, the standard solution (i.e. 100 μM) was made from 50 mL of distilled water, 0.01 g of ethylenediaminetetraaceitic acid (EDTA) and 0.011 g of S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP). SNAP is a NO donor and the efficiency is 60% when using Cu^{2+}. When the baseline is stable, known concentrations of SNAP (0-200 nM) were added to the copper sulfate to generate the current-NO concentrations curve and then calculated the calibration formula. To calibrate the H_2O_2 sensor, the standard (1

mM) H_2O_2 solution was then added to 10 mL of PBS in a range of 0-2 μM to generate the standard curve.

Animal Preparation

The Institutional Animal Care and Use Committee of Philadelphia College of Osteopathic Medicine approved all animal protocols performed in this study.

Once free radical sensor calibration is complete, male Sprague-Dawley rats ranging from 0.275-0.325 kg were anesthetized with 60 mg/kg pentobarbital sodium injections with 1000 unit heparin via intraperitoneal (i.p.). Maintenance anesthesia was given 30 mg/kg pentobarbital sodium (i.p.) as needed.

The animal preparation is shown in illustration figures 3 and 4. Initially, a PE-50 polyethylene catheter was inserted into the left carotid artery for monitoring mean arteriolar blood pressure throughout the entire experiment (see figure 3). Secondly, the jugular vein was catheterized (24 gauge catheter) superiorly to inferiorly in order to intravenously infuse different solutions, such as 20%, 30%, 50% D-glucose, 30% L-glucose, or saline (see figure 3). The 20%, 30%, 50% D-glucose, or 30% L-glucose solution was freshly made by dissolving D-glucose or L-glucose into saline. Different concentrations of D-glucose solutions were used to induce hyperglycemia at 200 mg/dL, 400 mg/dL, or 600 mg/dL, respectively. Due to the similar structure and weight as well as inability for the body to metabolize L-glucose, the 30% L-glucose served as an ideal control group for the 30% D-glucose.

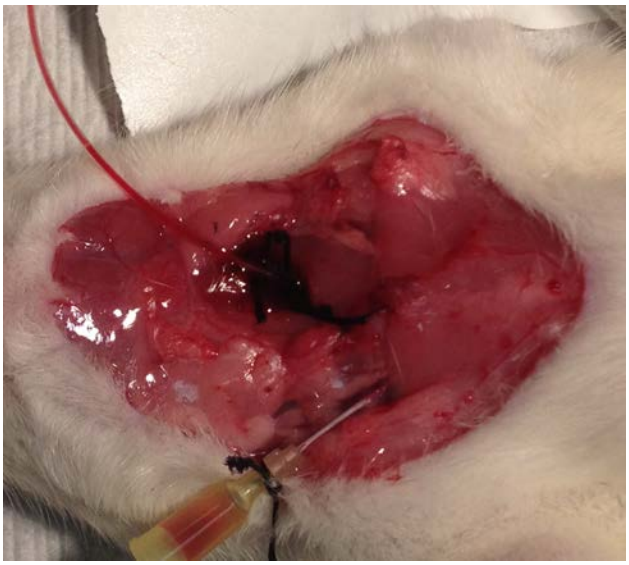


Figure 3. The catheterization of the jugular vein and carotid artery.

Both femoral veins were exposed and catheterized in order to place randomly a calibrated NO microsensor or a H_2O_2 microsensor into each respective femoral vein (see figure 4). These microsensors were connected to an Apollo 4000 monitor to measure blood NO and H_2O_2 levels in real-time. After 1 hour stabilization period to record baseline readings, saline or glucose solutions was administered at a rate of 0.25 mL/min for the first four minutes for loading dose, thereafter the infusion speed was maintained at rate from a range of 0.018 mL/min to 0.035 mL/min based on the target glucose level throughout the remainder of the experiment. Blood pressure, NO and H_2O_2 were recorded at the baseline and at 20 min intervals for a total of 180 min after intravenous infusion of saline, D-glucose or L-glucose. In every group, blood glucose was recorded from pricking the tail vein at the beginning and every 40 min through the experiment via Ascensia Contour blood meter. Moreover, respiratory rate and urination were observed to monitor the anesthesia status of the rats during the experiment.

Figure 4. Nitric oxide and hydrogen peroxide microsenors in the femoral veins.

After the experiment, the abdominal aorta and the vena cava were removed and stored in 4% paraformaldehyde for future analysis of leukocyte vascular adherence. The thoracic aorta was harvested and frozen for future immunohistochemistry or western blot analysis of adhesion molecules.

Furthermore, the current changes in NO or H_2O_2 release during hyperglycemia (in picoamps) were expressed as change relative to initial baseline. Thereafter, the picoamp values were converted to the concentration of NO (nM) or H_2O_2 (μM) after correction to the calibration curve of free radical microsensors. In order to further clarify the influence of D-glucose or L-glucose on blood NO and H_2O_2, data was also expressed as relative NO or H_2O_2 change under these conditions by subtracting the NO or H_2O_2 values of saline control group at each time point.

Experimental Groups

There were a total of 5 experimental groups in this study:

1.) Saline control group (n=7 for NO and n=5 for H_2O_2): The animal had the same surgery and was monitored the blood NO and H_2O_2 when saline was intravenously infused for 3 hours. This group was selected to show the animal can go through the surgery procedures and maintain stable conditions throughout the whole experiment period. Moreover, the level of blood NO and H_2O_2 in this group reflected the real-time vascular endothelial function and served as control to evaluate the change under hyperglycemic or hyperosmolarity conditions.

2.) Hyperglycemic group (200 mg/dL, n=7 for NO and n=6 for H_2O_2): The animal was intravenously infused with 20% D-glucose in order to maintain hyperglycemic condition at 200 mg/dl for 3 hours. This group was used to evaluate the real-time blood NO and H_2O_2 change at mild hyperglycemic conditions.

3.) Hyperglycemic group (400 mg/dL, n=6 for NO and n=6 for H_2O_2): The animal was intravenously infused with 30% D-glucose in order to maintain hyperglycemic condition at 400 mg/dl for 3 hours. This group was used to evaluate the real-time blood NO and H_2O_2 change at moderate hyperglycemic conditions.

4.) Hyperglycemic group (≥600 mg/dL, n=6 for NO and n=6 for H_2O_2): The animal was intravenously infused with 50% D-glucose in order to maintain hyperglycemic condition at ≥600 mg/dl for 3 hours. This group was used to evaluate the real-time blood NO and H_2O_2 change at severe hyperglycemic conditions.

5.) 30% L-glucose group (n=5 for NO and n=5 for H_2O_2): L-glucose is the enantiomer of D-glucose and cannot be synthesized or metabolized by living organisms as a source of energy. 30% L-glucose has the same osmolarity as 30% D-glucose. Therefore, this group serves as another control for the 30% D-glucose group to indicate if high osmolarity is also involved in the change of blood NO and H_2O_2 under

hyperglycemic conditions induced by D-glucose.

Statistics

All data in text and figures is represented as mean ± SEM. When comparing more than two groups, the analysis was done by ANOVA using post hoc analysis with the Bonfferoni/Dunn test to detect differences among experimental groups within each group. Probability values of <0.05 are considered to be statistically significant.

Results

The rats exhibited the normal vital signs for all groups throughout the experimental period. The blood pressure (BP) ranged from 90-130 mm Hg throughout the entire experiment except for the slight increase about 10-15 mmHg in blood pressure during the first 5 min of 50% D-glucose infusion. However, after 5 min, the blood pressure then subsided and remained stable throughout the experiment. In the hyperglycemic and L-glucose groups, the rat started to urinate from 20-40 min after glucose infusion to the 180 min of infusion.

Blood glucose levels in experimental groups

The blood glucose levels in every experimental group are shown in figure 5. The baseline of blood glucose ranged from 79-92 mg/dL and there was not significant among the groups. Infusion of saline for 180 min slightly raised blood glucose to 100±7 mg/dL at the end of the experiment.

Blood glucose was increased to 197±34 mg/dL after 20 min infusion of 20% D-glucose. Thereafter, the blood glucose was maintained around 200 mg/dL, showing 245±35 mg/dL at 60 min, 279±34 mg/dL at 100 min, 268±38 mg/dL at 140 min, and 247±25 mg/dL at 180 min of the continuous infusion. In 30% D-glucose infusion group, blood glucose was raised to 367±18 mg/dL after 20 min infusion. Thereafter, the blood glucose was kept around 400-500 mg/dL with continuous infusion. Blood glucose was 363±37 mg/dL at 60 min, 481±50 mg/dL at 100 min, 523±40 mg/dL at 140 min, and 428±30 mg/dL at 180 min of the continuous infusion. In contrast, the 30% L-glucose infusion did not dramatically increase blood glucose levels compared to 30% D-glucose, the blood glucose at 180 min was about 129±15 mg/dL. This indicated that the infusion

of L-glucose has no effect on changing blood glucose levels because L-glucose cannot be metabolized as D-glucose. The infusion of 50% D-glucose increased blood glucose to 549±22 mg/dL after 60 min infusion interval. The hyperglycemic condition was then remained at a range of 552 ± 20 mg/dL at 100 min, 600.0 ± 0 mg/dL at 140 min and 564 ±25 mg/dL at 180 min.

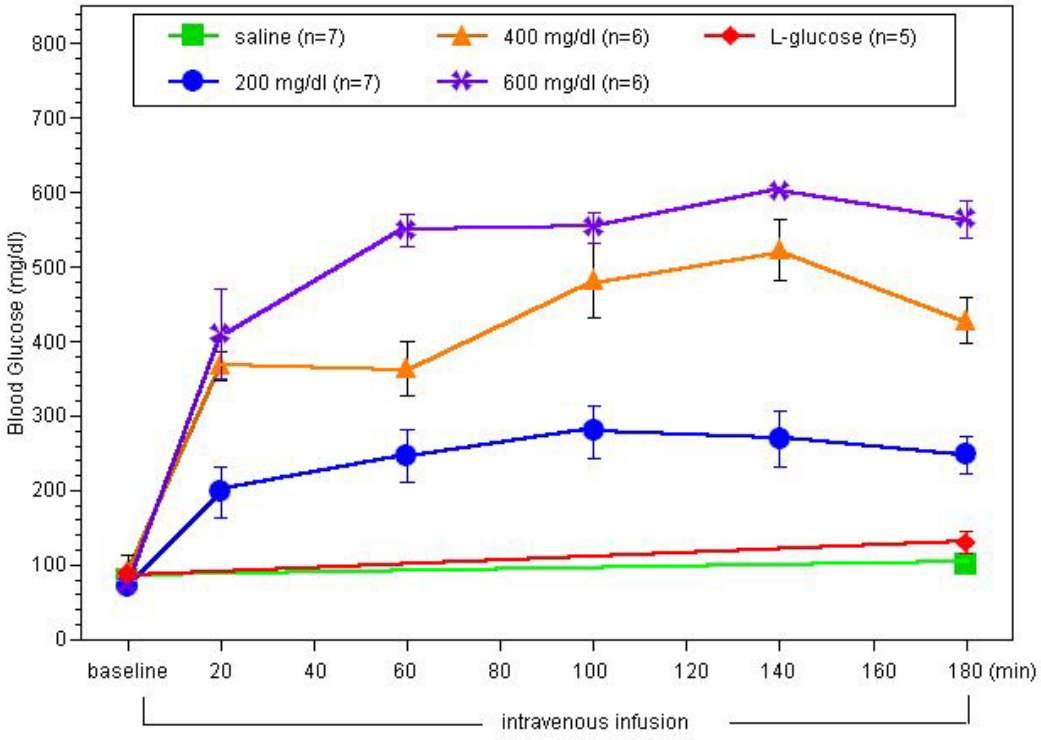


Figure 5. The comparison of blood glucose levels among saline, L-glucose hyperglycemia infusion groups.

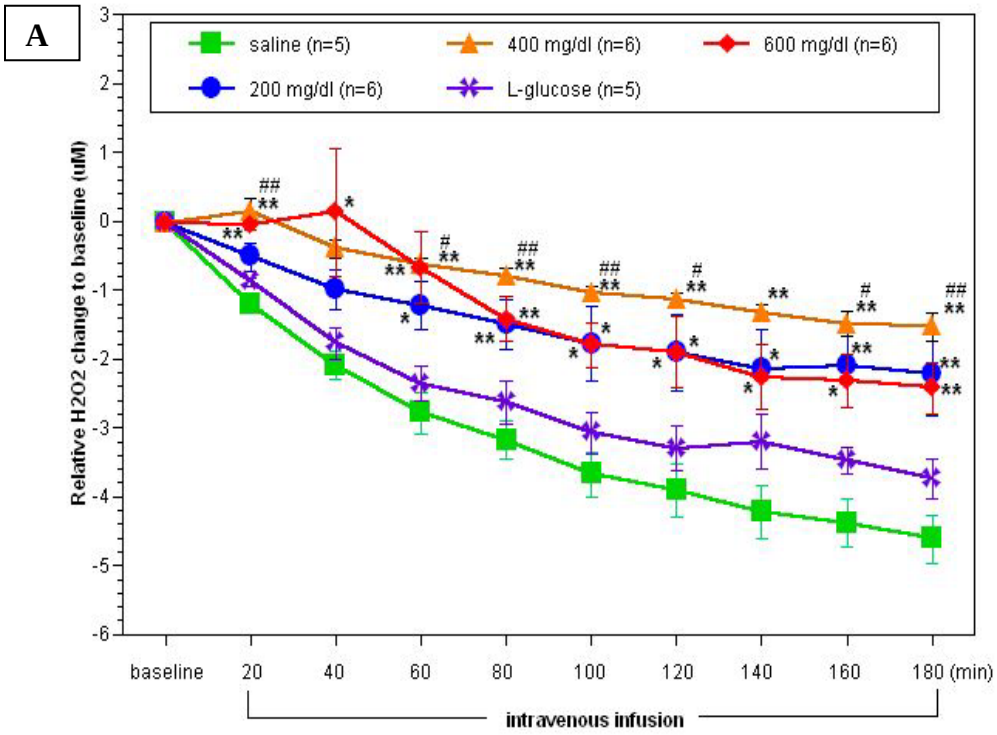
Blood H_2O_2 levels in experimental groups

Hyperglycemic conditions had significantly higher blood H_2O_2 levels compared to the saline control and L-glucose groups (Figures 6A and 6B). Figure 6A illustrates the H_2O_2 change relative to the baseline among different experimental groups. In the saline group, the blood H_2O_2 levels continued to drop and reduced by 4.62±0.34 μM (n=5) at the end of the 180 min infusion compared to the baseline. In the 200 mg/dL

hyperglycemic group, blood H_2O_2 levels stayed significantly higher at 60 min and remained significantly higher throughout the rest of experiment compared to saline group ($p<0.05$, figure 6A). Furthermore, the blood H_2O_2 in the 400 mg/dL hyperglycemic groups remained significantly higher at most of the experimental time points (i.e. 20, 60, 80, 100, 120, 140, 160, and 180 min) compared to saline group ($p<0.05$, figure 6A). Moreover, there was a dose-dependent effect in increasing blood H_2O_2 levels between these two hyperglycemic groups. At 180 min, blood H_2O_2 levels dropped by 2.22±0.61 μM (n=6) for 200 mg/dL group and by 1.53±0.20 μM (n=6) for 400 mg/dL group relative to baseline. In contrast, 600 mg/dL hyperglycemic group remained high blood H_2O_2 levels in the initial 40 minutes then quickly reduced to the similar H_2O_2 levels as that in 200 mg/dL group. There was no statistical significance between the 600 mg/dL and 200 mg/dL groups. At 180 min, blood H_2O_2 levels in 600 mg/dL hyperglycemic group dropped by 2.42±0.37 μM (n=6) relative to baseline. It was noted that blood H_2O_2 levels in 400 mg/dL hyperglycemic group ended up the highest out of all the groups. However, it was not significantly higher when compared to the other hyperglycemic groups. Blood H_2O_2 levels in L-glucose group were significantly lower compared to 400 mg/dL hyperglycemic group at most time intervals except for the 40 min and 140 min intervals ($p<0.05$). Moreover, this change was similar as saline control. At 180 min, blood H_2O_2 level in L-glucose group reduced by 3.75±0.29 μM (n=5) relative to baseline.

Figure 6B shows the time course of H_2O_2 change in hyperglycemic and L-glucose infusion groups relative to saline. Compared to saline control group, hyperglycemia significantly increased blood H_2O_2 levels at most of time points. By contrast, blood H_2O_2 levels in L-glucose group remained similar level (i.e. no statistical significant time points) as saline control throughout the experiment. At 180 min, blood H_2O_2 levels in 200

mg/dL, 400 mg/dL, and 600 mg/dL groups were 2.40±0.61 μM, 3.1±0.2 μM, and 2.21±0.37 μM higher than that in saline group, respectively (all $p<0.05$). The blood H_2O_2 levels were only 0.88±0.29 μM higher in the L-glucose group compared to the saline control at the end of the experiment.



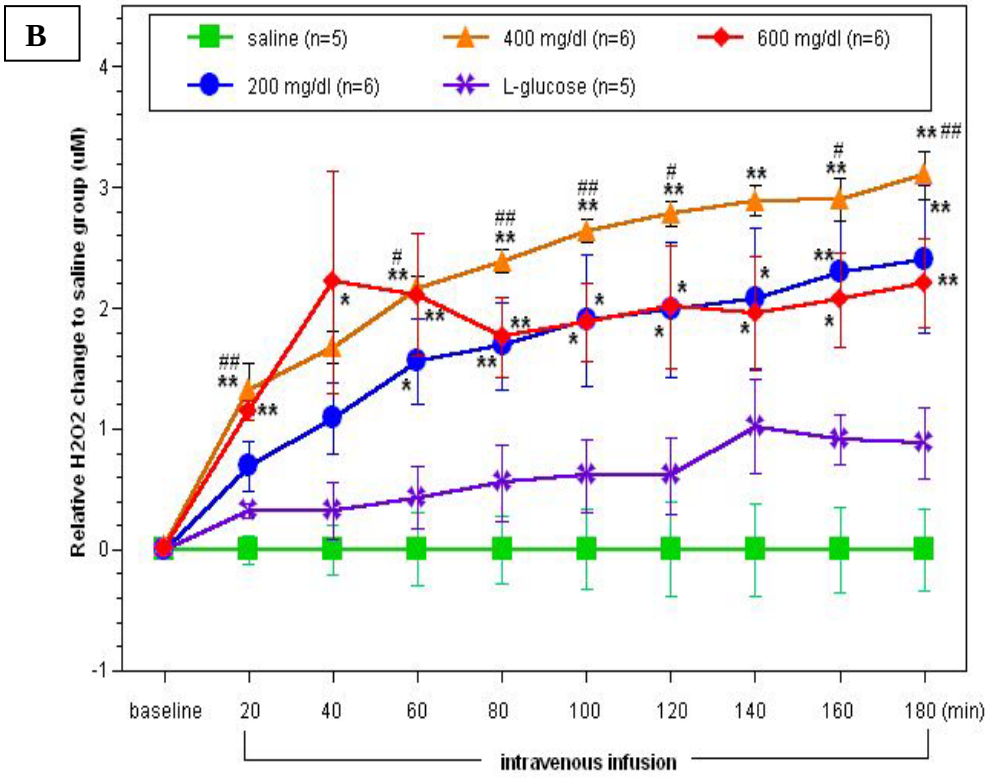


Figure 6A & 6B. The comparison of blood H_2O_2 levels among saline, hyperglycemia, and L-glucose groups relative to baseline (6A) or relative to saline group (6B). Hyperglycemic groups exhibited significantly higher blood H_2O_2 levels compared to saline control and L-glucose group (*p<0.05, **p<0.01 compared to saline; #p<0.05, ##p<0.01 compared to L-glucose group). The X axis indicates duration of experiment and the Y axis indicates the relative change of blood H_2O_2 in μM. The number of rats used for each group is indicated next to each labeled group.

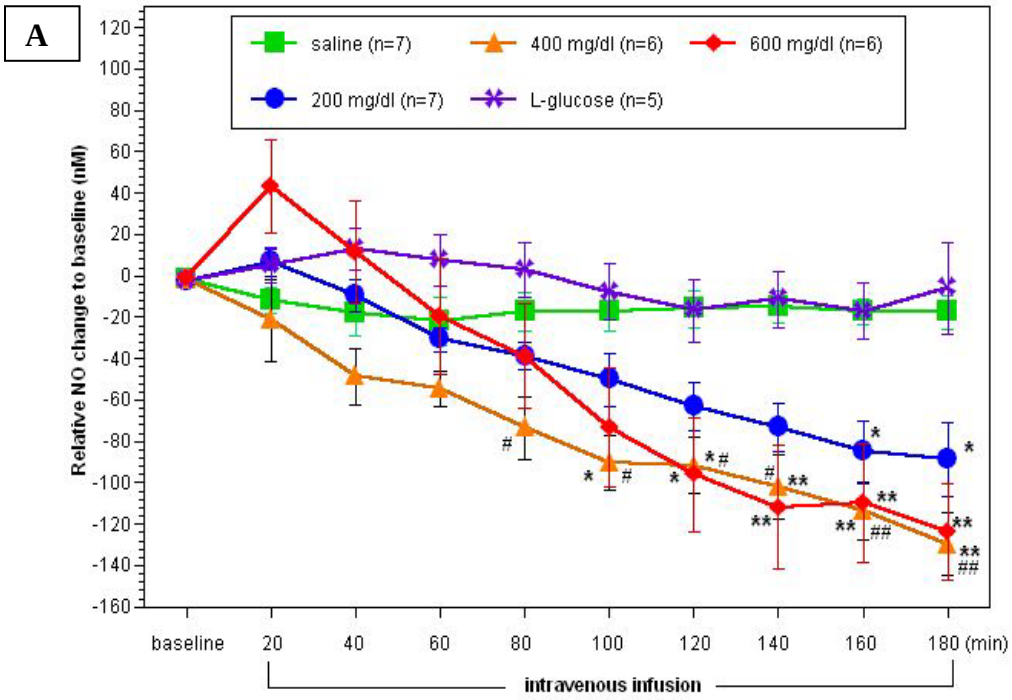
Blood NO levels in experimental groups

Hyperglycemic conditions had significantly lower blood NO levels compared to the saline control and L-glucose groups (Figures 7A and 7B). Figure 7A illustrates the blood NO levels relative to baseline among the different experimental groups. In the saline group, blood NO levels remained stable and only slightly decreased by 17.61±8.04 nM (n=7) at 180 min. In the 200 mg/dL hyperglycemic group, blood NO continued to drop and remained significantly lower level was noted at 160 min and 180 min of infusion compared to saline group (p<0.05, figure 7A). Furthermore, the blood NO in the

400 mg/dL hyperglycemic groups decreased immediately after infusion and remained significantly lower at 100 min, 120 min, 140 min, 160 min and 180 min compared to saline group ($p<0.05$, figure 7A). We observed a dose-dependent effect in reducing blood NO levels between the 200 mg/dL and the 400 mg/dL hyperglycemic groups. At 180 min, blood NO levels decreased by 88.91±17.90 nM (n=7) for 200 mg/dL group and by 129.76±15.28 nM (n=6) for 400 mg/dL group relative to baseline. By contrast, 600 mg/dL hyperglycemic group showed an initial NO increase at 20 min of infusion, then rapidly dropped below baseline at 60 min and remained significantly lower from 120 min to the end of the experiment compared to the saline control ($p<0.05$, figure 7A). At 180 min, blood NO levels in 600 mg/dL hyperglycemic group dropped by 123.59±23.45 nM (n=6) relative to baseline, which was similar (i.e. no statistical significance) to the NO levels in 400 mg/dL hyperglycemic group. Blood NO levels in L-glucose group were significantly higher compared to 400 mg/dL hyperglycemic group from 80 min to 180 min of infusion ($p<0.05$, see Fig 7A), which was similar as saline control in that there was not any statistical significance between the L-glucose and the saline control at any time point. At 180 min of infusion, blood NO levels in L-glucose group slightly changed by 6.03±22.01 nM (n=5) relative to baseline.

The blood NO levels were also analyzed by calculating the change in blood NO levels under hyperglycemia or L-glucose relative to saline control group at each time point (see figure 7B). Hyperglycemia significantly decreased blood NO levels at time points 100-160 min. There was no statistical significant difference between blood NO levels in L-glucose group and the saline control throughout the entire experiment. At 180 min, blood NO levels in 200 mg/dL, 400 mg/dL and 600 mg/dL groups were 71.3±17.9 nM, 112.15±15.28 nM and 105.98±23.45 nM lower than that in saline group, respectively

($p<0.01$). By contrast, the blood NO levels were only 11.58±22.01 nM (n=5) higher compared to the saline control at 180 min of infusion of 30% L-glucose.



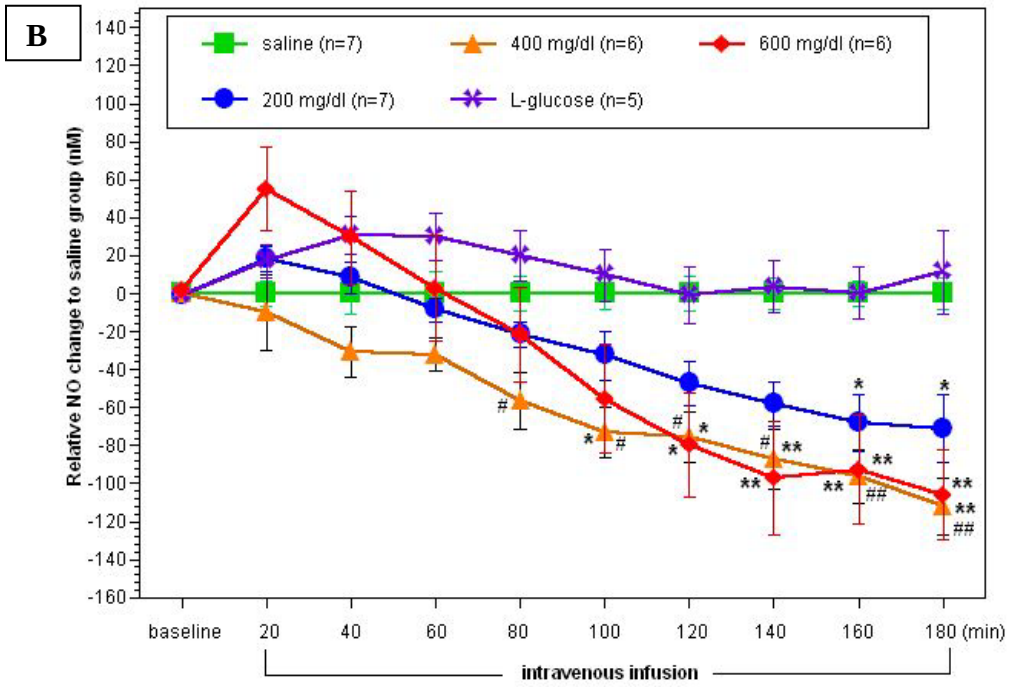


Figure 7A & 7B. The comparison of blood NO levels among saline, hyperglycemia, and L-glucose groups relative to baseline (7A) or relative to saline group (7B). Hyperglycemic groups exhibited significantly lower blood NO levels compared to saline control and L-glucose group (*p<0.05, **p<0.01 compared to saline; #p<0.05, ##p<0.01 compared to L-glucose group). The X axis indicates duration of experiment (i.e. time in minutes) and the Y axis indicates the concentration of blood NO in nM. The number of rats used for each group is indicated next to each labeled group.

Discussion

Summary of Results

The major findings of this study were: first, intravenous infusion of 20%, 30%, and 50% of D-glucose induced 200 mg/dL, 400 mg/dL, and 600 mg/dL hyperglycemia, respectively, and the different degree of hyperglycemia conditions was maintained for 180 min with continuous infusion. Second, under hyperglycemic conditions, blood H_2O_2 levels were significantly higher as compared to the saline control group. Third, by contrast, blood NO levels were significantly lowered under hyperglycemic conditions starting at 100 min through the rest of the hyperglycemic period as compared to the saline control group. Finally, 30% L-glucose exhibited significantly lower blood H_2O_2 and higher blood NO levels compared to 30% D-glucose, but no statistical significant difference was noted between this group and the saline control.

Acute Hyperglycemic rat model

We established a successful acute hyperglycemia induced vascular dysfunction rat model by measuring blood NO and H_2O_2 levels in real-time. The hyperglycemic rat model was based on the rat model used by Saha et al. (Saha, Xia et al. 2006), who showed that initial bolus injection of 20% glucose producing the maximum acute increase in blood glucose level (about 200 mg/dL). This increase was maintained as long as the rats received continuous glucose infusion (Saha, Xia et al. 2006). Therefore, we also used the bolus infusion of 1 ml D-glucose or L-glucose solution within 4 min for loading dose. Then the infusion rate was reduced by 10 fold to maintain the target blood glucose concentration throughout the 180 min experiment. Our blood glucose measurement showed that this infusion method can reach 200 mg/dL, 400 mg/dL or 600 mg/dL

hyperglycemic conditions around 20-60 min after D-glucose infusion and stayed relatively constant for the rest of experiment. In contrast, 30% L-glucose infusion did not cause high blood glucose which differed from the 30% D-glucose group and showed similar blood glucose levels as the saline group. Animals in all groups had relatively stable conditions with respect to the respiratory rate and BP. It was noted that there was initial increase of BP about 10-15 mmHg when the rats received the bolus injection of 50% D-glucose. This was maybe due to the activation of sympathetic nervous system responding to the excessively high concentration of D-glucose (Villafana, Huang et al. 2004). Under hyperglycemic conditions, rats urinated from 20-40 min to the end of infusion. This urination was also observed in L-glucose infused rats. Since 30% L-glucose had a similarly high osmolarity as 30% D-glucose, this data indicated that hyperosmolarity was the major cause of urination in the rats.

Increased Levels of H_2O_2 under Acute Hyperglycemic Conditions

We have demonstrated that H_2O_2 could serve as an indicator of the amount of SO produced under different pathophysiological conditions, such as ischemia-reperfusion (I/R) (Chen, Kim et al. 2010). In this study, H_2O_2 levels had dropped approximately 5μM within 180 min in the saline group. This phenomenon was most likely attributed to the decreased body temperature when the anesthetized rat was placed on a thermostatic board for almost 240 min. This H_2O_2 drop had also been observed in femoral I/R and lithotripsy rat models (Chen, Kim et al. 2010; Chen, Rueter et al. 2011). All hyperglycemic groups had a significantly higher blood H_2O_2 levels compared to saline control. The increased oxidative stress immediately occurred from the beginning of the intravenous infusion of high concentration D-glucose. Furthermore, the 200 mg/dL and 400 mg/dL

hyperglycemia groups demonstrated a dose-dependent effect on increasing blood H_2O_2 levels. This data was consistent with findings by Iori et al (Iori, Pagnin et al. 2008). Iori showed that the 20 mM glucose incubation induced a higher ROS production than the 10 mM glucose incubation cultured human endothelial cells.

Furthermore, we found that the 600 mg/dL hyperglycemia group had the highest H_2O_2 levels observed in only the first 20 min and then dropped off to levels similar H_2O_2 levels as 200 mg/dL at the end of the experiment. This may be due to the excessively high glucose, which may quickly damage the body system, such as causing cell death and inhibiting enzymes activity in the body. In Iori's experiment, he also found that the endothelial cells exposed to 20mM glucose had about 1.5 fold higher incidence of apoptosis as compared to the 10mM glucose group. Additionally, the activity of enzyme (i.e., heme oxygenase-1) was also reduced to a greater degree in 20 mM glucose (Iori, Pagnin et al. 2008).

The 30% L-glucose group, which has the same osmolarity as 30% D-glucose, exhibited significantly lower blood H_2O_2 levels compared to 400 mg/dL group which was infused with 30% D-glucose. The L-glucose is an isomer of glucose, and cannot be synthesized or metabolized by the body. Therefore, 30% L-glucose infusion group served as a control to test if hyperosmolarity also played a role in hyperglycemia induced blood H_2O_2 change. Our data suggests that high osmolarity caused by L-glucose infusion does not significantly contribute the hyperglycemia-induced oxidative stress. Similarly, Nakahata et al. also found that 20 mM D-glucose, not 20 mM L-glucose, significantly increased SO production in cerebral arteries after 60 min incubation (Nakahata, Kinoshita et al. 2008). This data supports the rationale that hyperglycemia-induced oxidative stress

and endothelial dysfunction are the subsequent events after D-glucose circulates in blood vessels and is utilized (Ding H and 2010).

Decreased Levels of Blood NO under Hyperglycemic Conditions

Blood NO levels in our saline group were relatively stable and only slightly dropped (by approximately 17 nM) relative to the baseline within the 180 min infusion period. The stability of blood NO in anesthetized rats had been observed in our femoral I/R and lithotripsy animal models (Chen, Kim et al. 2010; Chen, Rueter et al. 2011). In contrast to saline control, hyperglycemia significantly decreased blood NO levels in a dose-dependent manner between 200 mg/dL and 400 mg/dL. The 600 mg/dL hyperglycemia had an initial spike increase in NO production, and then quickly decreased when hyperglycemic condition was maintained. The initial increase of NO followed the BP increase caused by the bolus infusion of 50% D-glucose. Both events may be due to the activation of sympathetic nervous system to increase the secretion of epinephrine and norepinephrine, which further enhance BP and insulin secretion (Villafana, Huang et al. 2004).

Postprandial hyperglycemia has been highlighted in non-diabetic subjects because it can induce vascular endothelial dysfunction (Ceriello, Hanefeld et al. 2004). Kawano et al. found that the post-challenge hyperglycemia after oral glucose tolerance test rapidly suppressed endothelium-dependent vasodilation, probably through increased production of oxygen-derived free radicals in healthy subjects (Kawano, Motoyama et al. 1999). Our data by measuring blood NO levels provides clear evidence showing that acute hyperglycemia significantly reduces endothelial-derived NO bioavailability. The blood

NO measured during 180 min experimental period is not produced from iNOS because the synthesis of iNOS needs longer time than 180 min.

It is noted that there exists a negative relationship between H_2O_2 and NO. This is because increased superoxide production can directly quench NO. In our study, 400 mg/dL exhibited the highest blood H_2O_2 level associated with the lowest blood NO level. Moreover, the time course in significant changes of blood NO and H_2O_2 were different. H_2O_2 levels in the hyperglycemic groups (i.e., 400 mg/dL) were significantly higher at 20 min of infusion throughout the rest of 180 min experimental period compared to saline control. By contrast, NO levels in the hyperglycemic groups (i.e., 400 mg/dL) started a significant drop from 100 min to 180 min of the experiment compared to saline control. This discrepancy suggests that oxidative stress occurs relatively rapidly, then it will further reduce endothelial-derived NO levels. Cai et al. has showed that hyperglycemia resulted in a decrease in NO production by an increase in superoxide production which further causes BH_4 deficiency and eNOS uncoupling (Cai and Harrison 2000).

Mechanisms related to hyperglycemia-induced blood NO and H_2O_2 changes

Overview

A key factor to hyperglycemia-induced vascular endothelial dysfunction is oxidative stress. In this study we measured blood H_2O_2 levels and found that there existed oxidative stress under hyperglycemic conditions. It was suggested that the sources of oxidative stress under hyperglycemia include NADPH oxidase, uncoupled eNOS, and the mitochondria (Fonseca, Ravi et al.; Brownlee 2001; Forstermann and Munzel 2006). Moreover, all three sources were suggested to cumulatively produce excess SO in a state of hyperglycemia. Thereafter, SO will directly quench NO to form peroxynitrite and

reduce NO bioavailability. SO can also be further converted to H_2O_2 by SOD. Both peroxynitrite and H_2O_2 can cause eNOS uncoupling. Uncoupled eNOS will produce SO instead of NO to facilitate oxidative stress. Then, more SO can in turn uncouple more eNOS. This interaction between NO and oxidative stress may cause a vicious and detrimental cycle (see figure 8). This detrimental cycle can further cause vascular dysfunction, persistent inflammation, and tissue/organ damage. Furthermore, this cycle may provide a rationale to explain that H_2O_2 was significantly increased from the beginning of hyperglycemia, whereas NO did not significantly decrease until a later time of this experiment.

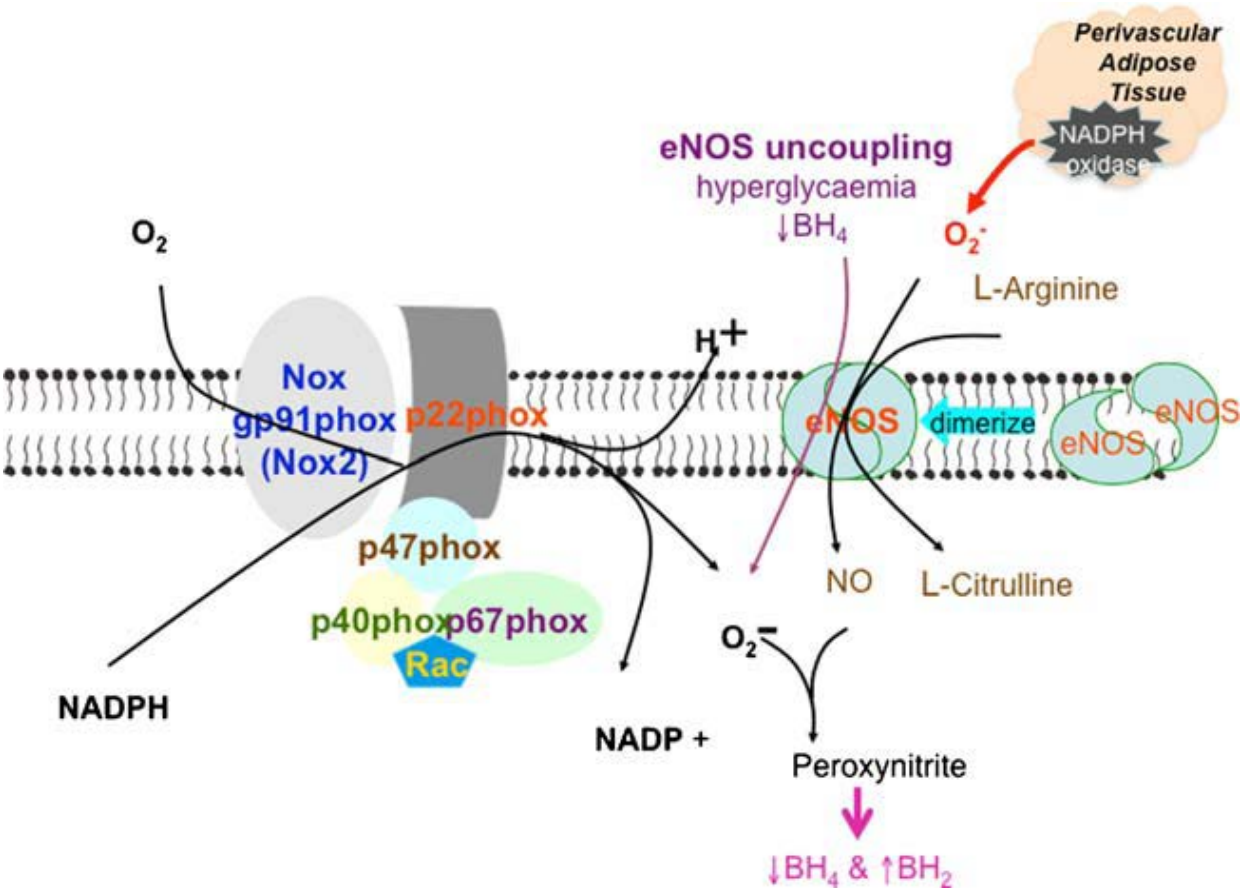


Figure 8. Model of vicious cycle under hyperglycemic conditions. An overview of the mechanism of how the generation of free radicals from hyperglycemia can cause a continuous production of more free radicals.

NADPH Oxidase

NADPH oxidase is a membrane bound and cytosolic enzyme producing superoxide by the utilization of NADPH. In addition to neutrophils, NADPH oxidase also presents in endothelial cells, vascular smooth muscle cells, and other cells. The physiological NADPH oxidase derived SO has been implicated in the regulation of vascular tone, vascular cell growth, migration, proliferation, and activation (Touyz 2003; Cai 2005). However, if NADPH oxidase is upregulated, higher amounts of SO may result in oxidative stress. Numerous studies have indicated that high glucose levels can activate the enzyme protein kinase C (PKC) in vascular cells. High glucose might stimulate SO

production through PKC-dependant activation of NADPH oxidase in vascular cells (Inoguchi, Li et al. 2000). Inogouchi et al. found that exposure of aortic endothelial cells to a high glucose level (400 mg/dL) for 72 hrs induced a significant increase in free radical production as opposed to cells that were exposed only to a glucose level of 100 mg/dL (Inoguchi, Li et al. 2000). Inogouchi also found that the increase in free radical production was negated through the use of diphenylene iodonium, a NADPH oxidase inhibitor (Inoguchi, Li et al. 2000). These results from Inoguchi suggest that high glucose levels may stimulate ROS production through the activation of NADPH oxidase. Furthermore, it has been found that SO production is significantly increased in streptozotocin-induced diabetic rats 2 weeks after the onset of diabetes. By contrast, NADPH oxidase inhibitor or PKC inhibitor can significantly attenuate this oxidative stress (Sonta, Inoguchi et al. 2004). It will be very interesting for us to investigate if a NADPH oxidase inhibitor can attenuate the blood H_2O_2 increase/NO decrease in this acute hyperglycemia model in the near future.

Uncoupled eNOS

Normally, in the presence of co-factor, BH_4, eNOS can keep electron transfer coupled with oxidation of L-arginine and then generate NO, which is defined as eNOS coupling (Schmidt and Alp 2007). Moreover, BH_4 and the amino acid L-arginine can stabilize eNOS dimer structure which has catalytic function (Fonseca, Ravi et al.). When BH_4 is oxidized to BH_2, BH_2 occupies the eNOS oxygenase domain, and blocks electron transfer to L-arginine, eNOS becomes uncoupled and dissociates to become a monomer, and then generates SO instead of NO (Vasquez-Vivar, Kalyanaraman et al. 1998). Fonseca et al. found that H_2O_2 can induce a collapse in the eNOS dimer resulting in

eNOS uncoupling (Fonseca, Ravi et al.). Additionally, the overproduced SO under hyperglycemia can bind NO to form the peroxynitrite. Peroxynitrite is very potent in oxidizing BH_4 to BH_2 resulting in decreased BH_4 to BH_2 ratio. Thereafter, eNOS becomes uncoupled and produce SO instead of NO (Crabtree, Smith et al. 2008). Thereby it is evident that a vicious cycle of oxidative stress under hyperglycemia can propagate: increased SO production directly reduces NO bioavailability by forming peroxynitrite. Moreover, peroxynitrite and H_2O_2 further cause eNOS uncoupling to produce SO instead of NO. Thereafter, more SO is generated, whereas less NO is produced. This process can keep repeating and can cause serious damage on vascular endothelial function. Therefore, uncoupled eNOS can be another source subsequent to oxidative stress induced by hyperglycemia to further exacerbate blood NO and H_2O_2 changes and vascular endothelial dysfunction. This vicious cycle can also help explain the time difference in blood NO and H_2O_2 significant changes found in this hyperglycemic study.

Mitochondrial SO production

Oxidative phosphorylation to generate ATP is the most prominent function of mitochondria. The system of oxidative phosphorylation includes five large multienzyme complexes. In normal physiological conditions and in most tissues, this system is an important source of SO. Meanwhile, SOD inside mitochondria can remove the SO to protect the mitochondria. Under hyperglycemia, it is hypothesized that there exists the increased hyperglycemia-derived electron donors from the TCA cycle (NADH and $FADH_2$), increases the mitochondrial membrane potential. This increased membrane potential inhibits electron transport at complex III, increasing the half-life of free-radical intermediates of co-enzyme Q, which reduce O_2 to SO (Brownlee 2001). This has been

proved by Srinivasan et al. They found that chronic exposure of human aortic endothelial cells to elevated glucose (25 mM) reduced total nitrite levels, eNOS mRNA and eNOS protein. This effect was reduced by the specific inhibition of reactive oxygen species production through the mitochondrial electron transport chain (Srinivasan, Hatley et al. 2004). These findings demonstrate that another possible source of SO under hyperglycemic condition is from the mitochondria.

D-glucose scavenging of NO

Moreover, in vitro, hyperglycemia can also be another factor in the significant decrease of NO. Brodsky et al. found that glucose can directly quench NO by the direct scavenging effect. He also correlated a link of diminished availability of NO in blood vessels when they were exposed to glucose. Furthermore, Brodsky provided experimental evidence showing that the healthy human subjects had a small, but significant reduction in NO-mediated vasoactivity when blood glucose was elevated to 270 mg/dL (Brodsky, Morrishow et al. 2001). This could serve as another possible factor to explain the drop in blood NO levels under acute hyperglycemic conditions.

Limitations/Future Studies

In this study, the blood NO and H_2O_2 levels were analyzed relative to the baseline or saline group. The free radical microsensor was calibrated before the experiment to test the sensitivity. Moreover, the correct poise voltage setup can also warrant the accuracy of the microsensors by defining the appropriate range for NO and H_2O_2 measurements. After the experiments, blood was collected and we plan to conduct fluorescence Griess Assay to measure the blood nitrite levels to confirm our NO data. Furthermore, blood

malondialdehyde concentration is also needed to reflect systemic lipid oxidation due to oxidative stress induced by hyperglycemic conditions.

We did appreciate that there may exist hyperinsulinemia in hyperglycemic rats. Our hyperglycemic rat model is induced in normal rats with normal insulin secretion responding to blood glucose change. It has been shown that hyperinsulinemia can cause less SO and more NO production. However, due to the continuation of D-glucose infusion, the excessive amount of D-glucose overrides on the effects of insulin to maintain hyperglycemic conditions. Therefore, the blood NO and H_2O_2 changes observed by this study can still indicate the detrimental effects on vascular function by hyperglycemia.

Furthermore, this is a pilot study to establish the acute hyperglycemic rat model and measure blood NO and H_2O_2 changes in real-time. We found that hyperglycemia maintained significantly higher H_2O_2 levels associated with significantly lower blood NO levels. It has been suggested that NADPH oxidase and eNOS uncoupling may be the important sources for these changes. Therefore, the further studies are needed to explore these related mechanisms by using NADPH oxidase inhibitor (diphenylene iodonium), or coupled eNOS cofactor (BH_4), or coupled eNOS substrate (L-arginine) with D-glucose infusion.

Conclusion

In summary, this study established an acute hyperglycemia-induced vascular dysfunction rat model by measuring blood NO and H_2O_2 levels in real-time. We found that acute hyperglycemia had a significantly higher blood H_2O_2 levels associated with significantly lower blood NO levels. The oxidative stress occurred immediately after

induction of hyperglycemia, whereas reduced endothelial-derived NO bioavailability took place at the later time points of hyperglycemia. This study indicates that oxidative stress is a predecessor of vascular endothelial dysfunction under hyperglycemic conditions and suggests that acute hyperglycemia for 180 min increased oxidative stress and reduced endothelial-derived NO bioavailability, which may not be due to high osmolarity.

Clinical Relevance

As stated in the introduction, hyperglycemia is linked to vascular endothelial dysfunction by increasing oxidative stress. Prolonged oxidative stress and vascular endothelial dysfunction most likely contribute to cardiovascular disease and atherosclerosis. The purpose of this pilot study was to establish a hyperglycemic model to test the relevant mechanisms to help prevent vascular dysfunction and other circulatory complications in diabetic and non-diabetic individuals. Hyperglycemia has been linked to the progression diabetic nephropathy. Hyperglycemia caused a production of reactive oxygen species that caused morphological and functional changes in mouse podocytes (Piwkowska, Rogacka et al.). It is also important to maintain normal glucose levels during surgery to avoid blood clots and stoke as well. Another factor that can cause hyperglycemia is psychological stress. To avoid future vascular complications, it is important for an individual to eliminate chronic psychological stress.

This could also bring awareness to the public about the hazards of postprandial hyperglycemia. It is a well known that consumption of excess dietary saturated fat and dietary sodium is directly linked to heart diseases. To promote better health, some individuals place some dietary restrictions on dietary fat and sodium. However, sugars

such as those found in soda, candy, etc. are not commonly seen through the eyes of the public as hazardous to heart health. It is known that a 20 oz bottle of the leading grape soda contains 81 g of sugar. When consuming a 20 oz bottle of this soda, one should anticipate similar vascular function changes as to the individuals in the 75 g post oral glucose challenge test mentioned in the introduction. This study provides the basic evidence for public to raise the awareness of the possible harmful impact of a high sugar diet on vascular function.

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