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The Role of Endothelial Nitric Oxide Synthase (eNOS) Coupling Status During Acute Hyperglycemia as Determined by Real-time Measurements of Blood Nitric Oxide And Hydrogen Peroxide in Rat

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A Thesis in Vascular Endothelial Dysfunction by Matthew Bertolet

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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.

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Abstract

Acute hyperglycemia can impair vascular endothelial function in non-diabetic subjects in addition to diabetic patients. Decreased endothelial-derived nitric oxide (NO) bioavailability and increased concentrations of reactive oxygen species (ROS), such as superoxide (SO) and hydrogen peroxide (H_2O_2), are the major characteristics of vascular endothelial dysfunction. Normally, vascular endothelial function depends on NO production from coupled endothelial NO synthase (eNOS) in the presence of 5,6,7,8-tetrahydrobiopterin (BH_4). By contrast, 7,8-dihydrobiopterin (BH_2, Oxidized form of BH_4) and/or lack of L-arginine (coupled eNOS substrate) causes eNOS uncoupling to produce SO, which can be quickly converted to H_2O_2. The role of eNOS uncoupling in acute hyperglycemia induced vascular dysfunction *in vivo* is unclear. In this study; we hypothesized that acute hyperglycemia (200 mg/dL) would increase H_2O_2 and decrease NO release in blood relative to saline control. By contrast, BH_4 or L-arginine would attenuate the acute hyperglycemia-induced blood NO and H_2O_2 changes. However, BH_2 will exacerbate the acute hyperglycemia-induced blood NO and H_2O_2 levels. To test the hypothesis, blood NO or H_2O_2 levels were measured simultaneously using calibrated NO or H_2O_2 microsensors (100 μm; WPI Inc.) by placing them into the femoral veins of male Sprague-Dawley rats. The electrical traces from microsensors were recorded at baseline and throughout 3 hours of infusion with saline or 20% D-glucose with or without a drug (BH_4,BH_2, or L-arginine) and converted into a concentration based on a calibration curve. Acute hyperglycemia (200 mg/dL by i.v. 20% D-glucose) significantly increased H_2O_2 (n=6) and reduced NO (n=6) blood levels in comparison to the saline group (n=7, $p<0.05$). BH_2 (MW=239.23 g/mol, 4mg/kg) exacerbated hyperglycemia,– induced

increased H_2O_2 levels (n=7) and decreased NO levels (n=6) ($p<0.05$). By contrast, BH_4 (n=6, MW=314.20 g/mol, 6.5mg/kg), significantly reduced blood H_2O_2 levels and increased blood NO levels during acute hyperglycemia compared to saline control ($p<0.05$). Moreover, L-arginine (MW=210.66 g/mol, 600mg/kg) had similar effects on H_2O_2 (n=5) and NO (n=6) blood levels as BH_4, showing significant reduction of blood H_2O_2 and enhancement of blood NO compared to saline control ($p<0.05$). In summary, uncoupled eNOS serves as a significant mechanism mediating acute hyperglycemia-induced vascular dysfunction and oxidative stress. Therefore, promotion of eNOS coupling may be effective in protecting vascular endothelial function from hyperglycemic insult.

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Introduction

Hyperglycemia and Vascular Complications

Hyperglycemia has been observed in association with many diseases such as diabetes, prediabetes, metabolic syndromes, and some post-surgery complications (13, 14, 28, 87). There is substantial evidence indicating that both acute and chronic hyperglycemia have negative effects on vascular function and are closely related to the development and progression of microvascular, neurologic, and macrovascular complications. The underlying mechanisms of hyperglycemia induced vascular/organ damage, however, are complex. It has been suggested that vascular endothelial dysfunction is a critical step in initiating the vascular damage. Therefore, it is vital to identify the mechanisms mediating hyperglycemia induced vascular endothelial dysfunction.

1. Clinical Hyperglycemic Conditions

Elevated fasting blood glucose is one of the approved criterion for diagnosing diabetes mellitus approved by the American Diabetes Association (ADA). This is measured by analyzing the plasma or serum glucose levels after at least 8 hours of fasting (usually done overnight). The range of normal human fasting plasma or serum glucose levels is between 70 and 100 mg/dL. A plasma glucose level of 126 mg/dL or greater indicates hyperglycemia. If the hyperglycemia occurs on more than one occasion after at least 8 hours of fasting, a diagnosis of diabetes mellitus is made. Prediabetes is defined when the fasting plasma glucose levels remain between 100 mg/dL and 125 mg/dL. Prediabetes can increase the risk for diabetes (30).

An oral glucose tolerance test (OGTT) is another method used to diagnose diabetes. For this test an adult is given a loading dose of 75 g of glucose in 300 ml of water. Blood samples are assayed for plasma glucose at the 0 minute and 120 minute mark after the loading dose is ingested. If the patient's blood glucose levels are greater than 200 mg/dL after the 120 minute mark a diagnosis of diabetes is made. An OGTT is considered normal if the two-hour blood glucose value falls below 140 mg/dL. If the two-hour postprandial blood glucose levels are between 140-199 mg/dL the patient is considered to have 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, polyuria, headaches, fatigue and blurred vision (27; 28; 30). Diabetic ketoacidosis (DKA) is a complication of diabetes mellitus that occurs when the body cannot use glucose as a fuel supply due to an insufficient amount of insulin in the body, and results in the breakdown of body fat for energy and, thus, an accumulation of ketones. DKA affects patients with serum glucose levels over 300 mg/dL. This severe hyperglycemia, if left untreated, can lead to coma and death. When blood glucose is 600 mg/dL or higher it can induce hyperglycemic hyperosmolar syndrome (HHS), an increase in blood viscosity due to the excess glucose in the blood and dehydration, which may result in coma and even death (52).

2. Acute Hyperglycemia in Non-Diabetic Subjects

In non-diabetic subjects, blood glucose levels can increase under some conditions such as following a high glucose meal (i.e., oral glucose tolerance test), stress, post-

surgery, certain medications, and critical illness (22; 25; 70). Fasting plasma glucose and postprandial glucose levels/OGTT often have been used clinically to indicate an acute hyperglycemia level (30).

Stress hyperglycemia is an acute, transient hyperglycemia caused by severe illness or post-surgery in patients/individuals without pre-existing diabetes (39). There is evidence that stress hyperglycemia in critically ill and preoperative patients can serve as an independent marker of complications such as: infection, a slower recovery time, and higher mortality (22; 25; 70). When hyperglycemia is uncontrolled it can lead to hypokalemia, hyponatremia, arrhythmias and an increased risk of ischemic brain injury (36). Hyperglycemia may also increase the risk of post-surgical infections in patients by impairing leukocyte functions including: reduced granulocyte adherence, impaired phagocytosis, delayed chemotaxis, depressed bactericidal capacity, and decreased neutrophil function (41; 67). Because of all of the consequences of hyperglycemia, it is important to make sure that patients maintain normal blood glucose levels at all times. It has been found that maintaining blood glucose under 110 mg/dL significantly reduces mortality and morbidity among critically ill patients both surgical and non-surgical treated critically ill patients (29).

Some studies have linked the role of acute hyperglycemia to the development of vascular complications in non-diabetic individuals (27). Furthermore, it has been shown that high dietary glucose consumption and postprandial hyperglycemia are associated with an increased risk of cardiovascular disease, even in the absence of clinically diagnosed diabetes (13). Hanefeld et al. have shown a link between postprandial hyperglycemia levels in nondiabetic patients and the incidence of increased intima-

media thickness (IMT), which is a generally accepted marker of early atherosclerosis (37). In a study containing 403 participants, Hanefeld noted that the carotid IMT was significantly increased in both the top quintile of 2 hour post prandial plasma glucose and peak plasma glucose concentration (37). Further indications were derived from pathophysiological studies which indicated that increased post prandial glucose levels are associated with increased production and difficult removal of triglyceride-rich lipoproteins, impaired fibrinolysis, and oxidative stress. These observations led Hanefeld et al. to suggest that postprandial hyperglycemia in conjunction with oxidative stress could have destructive effects on the arterial wall and may accelerate atherosclerosis (37). It also has been suggested that many cardiovascular risk factors are modified due to the increased postprandial glucose. One of the primary mechanisms could be that acute hyperglycemia may cause the overproduction of free radicals overwhelming the antioxidant mechanisms resulting in oxidative stress, which favors the development of vascular endothelial dysfunction and disease (20).

3. Chronic Hyperglycemia/Diabetes

Diabetes mellitus is the most common chronic metabolic disease and a major source causing morbidity and mortality (64). Diabetes mellitus is characterized by hyperglycemia due to a total or relative lack of insulin secretion and/or insulin resistance associated with different vascular complications (1; 3). In the year 2000, there were approximately 171 million diabetics and the prevalence is expected to increase up to 366 million globally by 2030 (85). As of today, the World Health Organization estimates that there are 347 million people worldwide who have diabetes (18). The long term prognosis

of those with diabetes remains poor due to the microvascular and macrovascular complications resulting from chronic hyperglycemia.

Microvascular complications from diabetes include retinopathy, nephropathy, and neuropathy. In 2011, the National Center for Chronic Disease Prevention and Health Promotion published data showing that diabetes is the leading cause of new cases of blindness and kidney failure among adults. Furthermore, about 60 - 70% of people with diabetes have mild to severe forms of nervous system damage. Moreover, greater than 60% of nontraumatic lower-limb amputations occur in people with diabetes (1). In terms of macrovascular complications, diabetes is characterized by a high prevalence of cardiovascular diseases (14). Diabetics have two to four times higher heart disease death rates than non-diabetic individuals (1). About 65% of patients with type II diabetes die from complications linked to heart disease or stroke (60; 68).

Postprandial hyperglycemia has been found to play an important role in the pathogenesis of vascular complications and cardiovascular disease (CVD) in diabetes. The study conducted by Meigs, Nathan et al showed that fasting hyperglycemia and 2 hour post-challenge hyperglycemia independently increase the risk for CVD, even after accounting for standard nonglycemic risk factors (59). Additionally, the "DECODE" study (Diabetes Epidemiology: Collaborative analysis Of Diagnostic criteria) in Europe indicated that the 2-hour glucose criterion alone increased the mortality risk of cardiovascular disease regardless of fasting plasma glucose levels (2). Postprandial hyperglycemia in a subset of type II diabetic patients has also been associated with myocardial perfusion defects, which might constitute a very early phase of the atherogenic process in the coronary circulation (73). Similarly, other studies have shown

that acute hyperglycemia increases the stiffness of intermediate-sized arteries and resistance arteries in young patients with type 1 diabetes (33).

Hyperglycemia and Vascular Endothelial Dysfunction:

It is vital to preserve the homeostasis of vascular endothelium in its resting state for proper blood perfusion into tissues and organs. Vascular endothelium can react with various physical and chemical stimuli and is involved in the pathogenesis of different diseases, such as ischemia/reperfusion injury, hyperglycemia, diabetes, and infectious diseases (65; 69). Hyperglycemia can disturb vascular endothelium homeostasis, induce vascular endothelial dysfunction, and initiate the cascade of vascular based organ dysfunction.

1. Normal Vascular Endothelial Function

The endothelium is composed of a monolayer of endothelial cells and lines the entire vascular system. Endothelial cell structure and functional integrity are essential in the maintenance of circulatory function via the vessel wall, which serves as a semi-permeable barrier and controls the transfer of small and large molecules (76). The vascular endothelium is normally maintained at a resting state and acts as a chief regulator of vascular homeostasis (84). Endothelial cells can produce and release a variety of vasoactive substances including prostacyclin and nitric oxide in response to chemical stimuli and alterations in hemodynamic forces (31; 45; 76). Consequently, under standard conditions vascular endothelial cells maintain an antithrombotic surface

which facilitates adequate blood flow by regulating thrombosis, thrombolysis, platelet and leukocyte adherence, and vascular tone (31; 76; 92).

Nitric Oxide

Nitric Oxide (NO), a potent endogenous vasodilator, is the most important vasoactive substance produced by the vascular endothelium (19). It serves as a vasodilator to all blood vessels in the body and reduces inflammation. Under normal physiological conditions, endothelial derived NO is produced by the membrane bound enzyme endothelial nitric oxide synthase (eNOS). eNOS oxidizes the amino acid L-arginine in a five electron oxidation step to form the intermediate N^G-hydroxy-L-arginine (34). Reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) and molecular oxygen are two important substrates, while BH_4 is an essential cofactor in this reaction (42; 82). The oxygenase domain of eNOS contains a BH_4 prosthetic group. By donating a single electron, BH_4 activates heme-bound oxygen which is then recaptured to enable NO release (94). It has been indicated that L-arginine and BH_4 are two critical factors in stabilizing the dimerization of the eNOS structure for electron transfer and NO production. Accordingly, 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) (72).

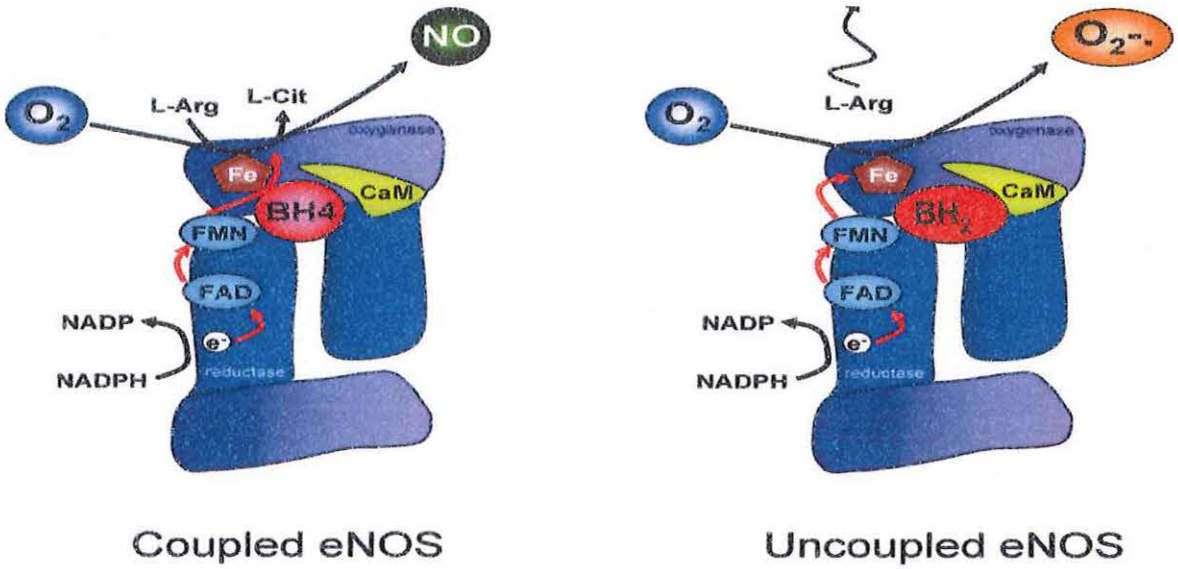

Figure 1. Coupled and uncoupled eNOS activity. Endothelial NOS coupling occurs using BH_4 as a cofactor during a physiological condition allowing the reaction to produce NO and L-citrulline from L-arginine /NADPH/O_2. When uncoupled, eNOS uses BH_2 as a cofactor to produce SO. Picture is adapted from (72).

The synthesis and release of NO occur in response to stimuli acting on the endothelial cell surface. This is dependent on the increase in intracellular calcium levels resulting in calmodulin formation, which leads to displacing caveolin and activating the eNOS (44). The release of NO can be stimulated by physical forces, such as the shear stress of flowing blood, and neurohumoral factors, such as acetylcholine or bradykinin (82). As a strong and lipophilic gas NO is able to permeate cell membranes freely and diffuse toward smooth muscle cells and into the vascular lumen (See figure 2) (84).

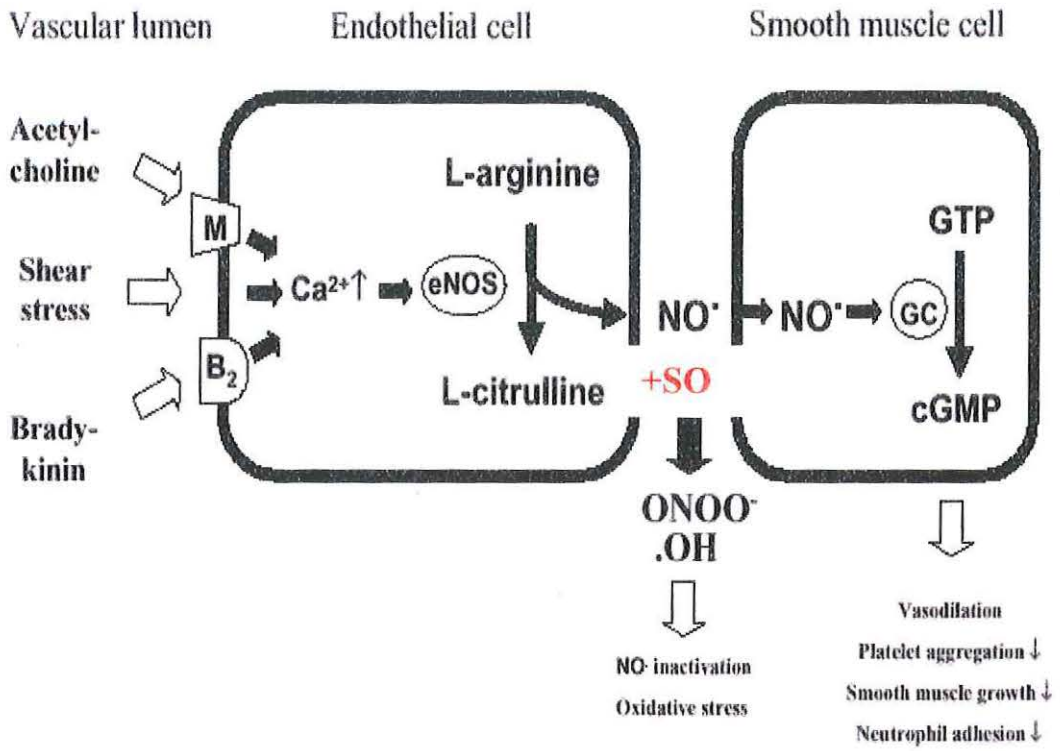

Figure 2. The production of NO in vasculature and its major physiological and pathophysiological effects. Picture is adapted from (61).

NO exerts its effect in smooth muscle cells by binding to the prosthetic heme group of the NO-sensitive guanylyl cyclase enzyme (4). Activated NO-sensitive guanylyl cyclase (GC) results in an increased production of cyclic 3'5' guanosine monophosphate (cGMP) from GTP (5). cGMP is an important second messenger which results in the activation of cGMP-dependent protein kinase, which further lead to the dephosphorylation of myosin light chain kinase (MLCK). K^+ channels have been shown to be phosphorylated by cGMP-dependent protein kinase, leading to the membrane hyperpolarization and reduction of intracellular Ca^{2+} ions. Together, these collective processes result in the relaxation of smooth muscle cells (See figure 2) (44; 50).

In addition to its function as a vasodilator, NO has numerous effects on the cardiovascular system which include the reduction of small amounts of SO radicals, as well as an inhibitory effect on platelet aggregation and smooth muscle cell proliferation (47). NO plays an essential role in preventing neutrophil and platelet adhesion, aggregation, and activation (See figure 2) (31). Kubes et al. demonstrated that inhibiting the synthesis of NO production by using N^G monomethyl-L-arginine (L-NMMA) and N^G nitro L-arginine methyl ester (L-NAME), analogues of L-arginine that inhibit NO production, resulted in a 15-fold increase in leukocyte adherence and a significant increase in transmigration of leukocytes in cat mesenteric postcapillary venules (46). These transmigrated leukocytes can ultimately release SO, myeloperoxidase, elastase, and lipases which can cause cell and tissue damage in pathology (48; 90). These results show that endothelial-derived NO is a significant endogenous inhibitor of leukocyte-endothelial interactions and adhesion (46).

There are two other isoforms of NOS enzyme in addition to eNOS. Each of the NOS isoforms has specific functions. Neuronal NOS (nNOS) is found in the nervous system as well as skeletal muscle, cardiac muscle and smooth muscle (89). nNOS constitutes the predominant source of NO in neurons and is localized to synaptic spines. Inducible NOS (iNOS) is found in the immune system and utilized by macrophages, neutrophils, and other inflammatory cells. iNOS expression increases during the inflammatory process and some malignant transformations (27; 43). Neuronal NOS and eNOS are constitutively expressed and Ca^{2+}/calmodulin-dependent. Both nNOS and eNOS produce small amounts of NO (i.e., nM range), which has precise actions on adjacent cells (89). In contrast, iNOS is Ca^{2+}/ calmodulin independent and produces high

levels of NO (i.e., μM range) in response to inflammatory cytokines and other stimuli (62).

As previously stated, NO diffuses into smooth muscle activating the NO-soluble GC, however, the majority of the NO that does not diffuse abluminally reacts rapidly with hemoglobin to form nitrate and methemoglobin if oxygenated, and iron-nitrosylhemoglobin if deoxygenated. Approximately 20% of NO produced by eNOS in the vasculature escapes inactivation by hemoglobin and becomes oxidized to nitrite (NO_2^-) in the plasma by a ceruloplasmin catalyzed reaction, and functions to modify proteins and lipids to form low concentrations of *N*-nitrosamines, *S*-nitrosothiols (RSNO), and nitrated lipids. Nitrite may also function as a stable vascular store of NO that can be enzymatically reduced to NO by deoxygenated hemoglobin along the physiological oxygen and pH gradient (51).

Reactive Oxygen Species (ROS)

ROS are molecules derived from molecular oxygen including SO, H_2O_2, and the hydroxyl radical ($^{\cdot}HO$) (35). Some of the potential sources of ROS include uncoupled eNOS, NADPH oxidases, xanthine oxidase, the cytochrome P-450 enzymes, the mitochondrial electron transport chain, the arachidonic acid metabolizing enzymes lipoxygenase and cycloxygenase, peroxidases, and other hemoproteins (10; 35). Under normal conditions, ROS have beneficial effects at low concentrations and encompass physiological roles in host defense mechanisms (against infectious agents) and in a number of cellular signaling systems (87). Furthermore, a delicate balance exists between the formation of these oxidizing substances and their effective removal by defensive

antioxidant mechanisms under normal physiological conditions (31). As a very unstable compound, SO has a half-life of seconds. Superoxide dismutase (SOD) converts SO to H_2O_2. Subsequently H_2O_2 is converted to water by catalase to facilitate its removal from the body. Because of the half-life of H_2O_2 is several minutes and more accurate to measure, the measurement of H_2O_2 will therefore serve as a good indicator of blood SO production (16).

2. Endothelial Dysfunction and Hyperglycemia

The dysfunction of vascular endothelium has been recognized as an essential component in vascular/organ damage because it is a common and early feature (57; 71). In much of the literature, endothelial dysfunction has been defined as the impairment of endothelium-dependent vasorelaxation caused by the loss of NO bioactivity in vessel walls (11). It also has been associated with increased oxidative stress (93). High-frequency ultrasonographic imaging of the brachial artery is a noninvasive approach that utilizes the ultrasound to measure blood vessel diameter and blood flow in order to assess endothelium-dependent flow-mediated vasodilation (FMD) (14; 56).

Hyperglycemia reduces the bioavailability of NO resulting in the development of vascular endothelial dysfunction (17). There is a great amount of evidence that suggests that vascular endothelial dysfunction is one of earliest events induced by hyperglycemia. The OGTT on healthy subjects with normal glucose tolerance, which simulates postprandial hyperglycemia, resulted in a significant reduction in flow-mediated endothelium-dependent vasodilation at 1 hour, and by 2 hour post challenge was restored to the baseline (40). Correspondingly, the postprandial hyperglycemia in relatively

healthy subjects can acutely affect endothelium-dependent regulation of blood flow (81). Furthermore, application of BH_4 to these healthy subjects reverses postprandial hyperglycemia-induced vascular endothelial dysfunction at the 1 hour mark (81). Conversely, the recovery of the flow-mediated endothelium-dependent vasodilation response post-challenge was greatly diminished in diabetic patients who have chronic hyperglycemia (40).

Endothelial NOS and Hyperglycemia

As previously stated, eNOS coupling requires the presence of the substrate L-arginine and cofactor BH_4. Conversely, when L-arginine is insufficient or BH_4 is oxidized to BH_2 which in turn reduces the ratio of BH_4 to BH_2, eNOS becomes "uncoupled" and uses molecular oxygen to produce SO instead of NO (See Figure 1) (17). While it is suggested that eNOS "uncoupling" can mediate vascular endothelial dysfunction under hyperglycemia conditions, it is still uncertain that eNOS "uncoupling" is attributed to the lack of L-arginine substrate or the reduced BH_4 to BH_2 ratio (17).

Oxidative Stress under hyperglycemia

Oxidative stress usually refers to the process of cellular damage as a consequence of the uncontrolled action of ROS (84). This damage occurs due to the imbalance between the over formation of oxidizing agents (ROS) and reduced removal by antioxidants in favor of ROS accumulation (31). Pathologies caused by SO are unfavorable and well recognized (23). ROS such as SO have been shown to initiate cell death programs such as apoptosis and cell necrosis. Additionally, they have been shown

to impair vasodilatory responses of the vascular endothelium and oxidize proteins and lipids, and damage DNA (31; 49). Moreover, SO can combine with NO and inactivate the bioavailable NO at a rapid rate of 6.7×10^9 $mol/L^{-1}\text{-}s^{-1}$ (78). The new compound produced is called peroxynitrite ($ONOO^-$), which is very cytotoxic and induces cell damage by oxidizing BH_4 to BH_2. With a decreased BH_4 to BH_2 ratio, uncoupling of eNOS results in an increased production of SO (See Figure 1). Subsequently, endothelial dysfunction can be induced and serves as a feedback inhibition of NO production (31).

Levels of ROS, particularly SO, significantly increase when endothelial cells are exposed to high glucose (28; 91). Studies have shown that systemic oxidative stress and lipid oxidation occur under both acute and chronic hyperglycemic conditions (58). The enzyme NADPH oxidase, in addition to uncoupled eNOS, serves as another important source of SO production in vascular tissue. Under hyperglycemic conditions, protein kinase C- beta (PKC- β) is activated which further induces NADPH oxidase activation to produce the superoxide anion. PKC- β inhibitors have been shown to prevent the hyperglycemia induced decrease in endothelium-dependent vasodilation (7). Moreover, xanthine oxidase, NADPH oxidase of neutrophils, and mitochondrial dysfunction also serve as sources for the production of SO. Some studies have shown anti-oxidants, such as vitamin C, attenuated the abnormality in endothelium-dependent vasodilation caused by hyperglycemia (6).

Vascular endothelial dysfunction can further initiate leukocyte-endothelial interactions. Vascular endothelial adhesion molecules such as P-selectin, intercellular adhesion molecule-1(ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) are upregulated to promote leukocyte rolling, adherence and transmigration (63).

Furthermore, transmigrated leukocytes cause further inflammatory reactions by releasing cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) to recruit more leukocytes, and by releasing proteases and ROS which can cause endothelial injury. Organ and tissue damage can be induced from vascular endothelial dysfunction associated inflammation (66).

Summary of Background and Hypothesis

It has been established that chronic hyperglycemia induces oxidative stress and diminishes endothelial-derived NO bioavailability, which are closely associated to microvascular and macrovascular complications of diabetes. Multiple studies suggest that acute hyperglycemia, especially postprandial hyperglycemia, may increase oxidative stress and cause vascular endothelial dysfunction in healthy individuals. Acute hyperglycemic exposures that ranged from one to six hours impair endothelium dependent vasodilatation and can cause acute oxidative stress in healthy nondiabetic subjects (6; 79; 86). Furthermore, various studies suggest that acute hyperglycemia can decrease the BH_4/BH_2 ratio and increase asymmetric dimethylarginine concentrations. The reduced BH_4/BH_2 ratio facilitates eNOS uncoupling. Asymmetric dimethylarginine interferes with the L-arginine in the production of NO via eNOS. It has been suggested that eNOS uncoupling is involved in the vascular function change resulting in decreased NO bioavailability and increased ROS production in vivo in non-diabetic subjects, respectively (17; 53).

Conversely, in a study done by McNulty on nondiabetic subjects, it was found that a single episode of acute hyperglycemia for 1 hour causes systemic oxidative stress and lipid oxidation. However, neither acetylcholine (ACh)-mediated coronary endothelial NO release nor the subsequent bioavailability, metabolism, or action of endothelium-derived NO within the coronary circulation was reduced. This may be due to the short length of the experiment (58). For these studies, the researchers used ACh or other vasodilators to evaluate vascular dilatory function. Furthermore, they did not measure blood NO and H_2O_2 changes in real time. Moreover, there is a lack of knowledge that hyperglycemia induced eNOS uncoupling is due to reduced BH_4/BH_2 ratio or insufficiency of L-arginine (17; 53). None of the above experiments utilized eNOS substrates (eg. L-arginine), eNOS coupling cofactor (eg. BH_4), or eNOS uncoupling factor (eg. BH_2) to evaluate blood NO and H_2O_2 in real-time in-vivo.

Our lab has established a novel method to measure blood NO and H_2O_2 from rat femoral veins in real-time (16; 61; 77). Minni et al. found that acute hyperglycemia induced higher H_2O_2 and lower NO levels in blood, which is consistent with acute hyperglycemia-induced vascular dilatory dysfunction and systemic oxidative stress found in other studies.

In order to further clarify the role of eNOS uncoupling on vascular endothelial function under hyperglycemic conditions, we plan to monitor blood NO and H_2O_2 in real time under three hours acute hyperglycemia, and with supplementation of BH_4, BH_2, or L-arginine to test the following hypotheses:

1. Acute hyperglycemia (200 mg/dL) will increase H_2O_2 and decrease NO release in blood relative to saline control.

2. BH_4 (MW=314.20 g/mol, 6.5 mg/kg) or L-arginine (MW=210.66 g/mol, 600 mg/kg) will attenuate acute hyperglycemia-induced blood NO/H_2O_2 change.

3. BH_2 (MW=239.23 g/mol, 4 mg/kg) will exacerbate acute hyperglycemia-induced blood NO/H_2O_2 change.

This study aims to provide novel evidence regarding the contribution of eNOS uncoupling to SO production and reduced NO bioavailability during acute hyperglycemia. Furthermore, this study will provide potential treatments to improve vascular function and to prevent further tissue/organ damage under acute hyperglycemic conditions.

Methods

NO or H_2O_2 Microsensor Calibration

Prior to surgery, the NO or H_2O_2 microsensors (100 μm, World Precision Instruments (WPI), Sarasota, FL) were calibrated using known concentrations of NO and H_2O_2 solutions as per the manufacturer's recommendations. The selective membrane covering the sensor dictates the specificity of the free radical microsensors. NO or H_2O_2 in the solution or biological fluid diffuses through the membrane and is oxidized at the working electrode. At this point an electrical (oxidation/reduction) signal is generated with amplitude proportional to the free radical concentration of each specific sample. The different sensors have a selective poise voltage important for obtaining reliable data. A high performance Faraday shield incorporated in the sensor is used to minimize environmental noise (88).

A standard curve was constructed based on electrical changes responding to known concentrations of the free radical of interest. This enabled a conversion of the electrical signal recorded during the experiment to a molar concentration of NO or H_2O_2. To retrieve a baseline, the NO and H_2O_2 microsensors were soaked in 10 mL copper sulfate and 10 mL of PBS respectively. The microsensors were connected with cables to the Apollo 4000 free radical analyzer (WPI, Sarasota, FL). A standard solution (i.e. 100 μM) made from 100 mL of distilled water, 0.0022 g of S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP), and 0.002 g of ethylenediaminetetraaceitic acid (EDTA) was used to calibrate the NO microsensor. SNAP is a NO donor and has an efficiency of 60% to convert into NO when using copper sulfate (Cu^{2+}). Once the baseline recording of the NO microsensor became stable, known concentrations of SNAP (0-200 nM) were added

to the copper sulfate to generate the current-NO concentration curve and derive the calibration formula. To calibrate the H_2O_2 sensor, a standard (1 mM) H_2O_2 solution was then added to 10 mL of PBS in a range of 0-2 μM to generate a standard curve and derive the corresponding calibration formula. The respective NO and H_2O_2 sensor calibration formula was used to convert the experimentally measured electrical change to a blood NO (nM) and H_2O_2 (μM) concentration change.

Animal Preparation

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

Once calibration of the free radical sensors was complete, male Sprague-Dawley rats (Charles's River: Charles's River, Kinston, NC) ranging from 0.275-0.325 kg were anesthetized with 60 mg/kg of 50 mg/ml pentobarbital sodium injections and anti-coagulant 1000 unit heparin via intraperitoneal (i.p.) injections. A maintenance dose of 30 mg/kg pentobarbital sodium (i.p.) was administered as needed to maintain anesthesia throughout the procedure.

The animal preparation is illustrated figures 3 and 4. Initially, the jugular vein was catheterized (24 gauge catheter) superiorly to inferiorly for introducing the following control or experimental solutions after establishing a baseline. The 20% D-glucose solution was freshly made by dissolving 3g of D-glucose into 15 ml of 0.9% sodium chloride in order to introduce hyperglycemia at 200 mg/dL, for 3 hours after a loading dose of 20% D-glucose 0.25 ml/min for 4 minutes. BH_4, BH_2, and L-arginine were added

to 20% glucose to reach approximately 250 μM, 200 μM, and 27.6 mM in blood, respectively. Saline infusion served as the control.

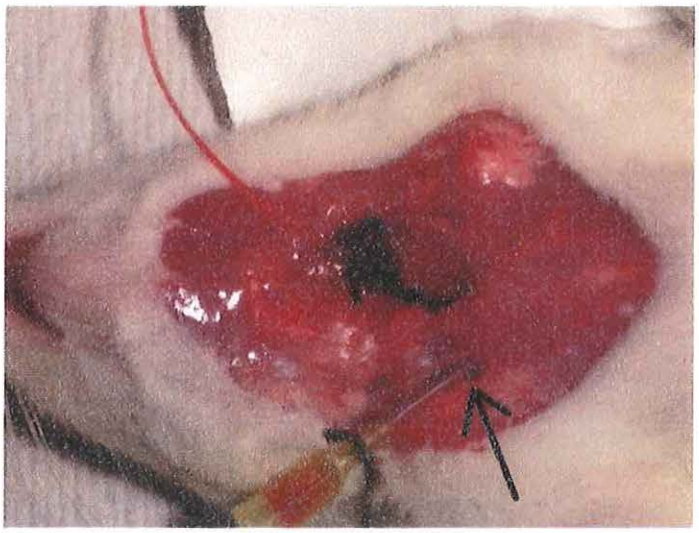

Figure 3. The catheterization of the jugular vein as arrow indicates.

Both femoral veins were exposed and catheterized in order to place a calibrated NO microsensor in one femoral vein and a calibrated H_2O_2 microsensor in another at random (see figure 4). These microsensors were connected to an Apollo 4000 monitor to measure blood NO and H_2O_2 levels in real-time. Baseline readings of blood NO and H_2O_2 were established after about an hour stabilization period. Then the saline or other treatment solutions (i.e. 20% D-glucose, 20% D-glucose with 6.5 mg/kg BH_4, 20% D-glucose with 4 mg/kg BH_2, or 20% D-glucose with 600 mg/kg L-arginine (see figure 3) were administered at a rate of 0.25 mL/min for the first four minutes for a loading dose. Subsequently, the infusion speed was adjusted at rates from a range of 0.014 mL/min to 0.025 mL/min to maintain 200 mg/dL hyperglycemia level throughout the remainder of the experiment for any test solutions containing 20% D-glucose. Infusion speed was maintained at 0.025 mL/min for the saline control group. Blood NO and H_2O_2 were

recorded continuously at 20 min intervals for a total of 180 min after intravenous infusion of different treatment/control solutions. In every group, blood glucose was recorded from tail vein samples at the beginning and every 20 min through the experiment via an Ascensia Contour blood glucose meter. Respiratory rate was monitored as an indicator of the level of anesthesia throughout the procedures.

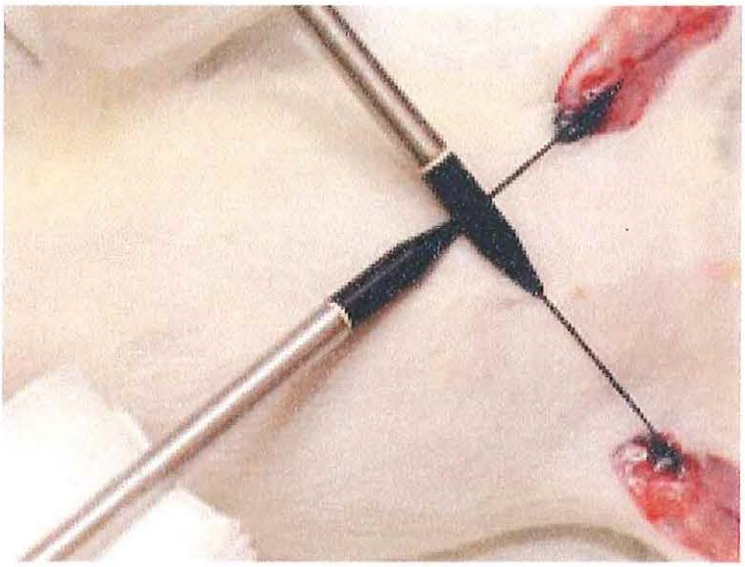

Figure 4. NO and H_2O_2 microsensors in the femoral veins.

After the experiment was completed, the animal was euthanized while maintaining anesthesia by opening the thoracic cavity through the diaphragm and transecting the aortic artery and vena cava. These were immediately removed along with the abdominal mesentery and one kidney and placed in 4% paraformaldehyde for future histochemistry analysis. The heart, thoracic aorta, and remaining kidney were harvested and frozen for future western blot analysis of adhesion molecules and blood plasma was collected for future analysis of serum cytokines and nitrite levels.

The current changes in NO or H_2O_2 release during the experiment (in picoamps) were expressed as change relative to the baseline. Subsequently, the blood NO and H_2O_2 recording in picoamp values were converted to the concentration of NO (nM) or H_2O_2 (μM) according to the corresponding calibration formula for the free radical microsensors previously generated. In order to further clarify the influence of 20% D-glucose, 20% D-glucose with BH_4, 20% D-glucose with BH_2, and 20% D-glucose with L-arginine 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 for the 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=6 for NO and n=7 for H_2O_2): The animals had the same surgery and were monitored for blood NO and H_2O_2 concentration changes when saline was intravenously infused for 3 hours following a one hour baseline period. This group was designed to show that the animal can undergo the surgery 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 conditions.

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

3.) Hyperglycemia + BH_4 [MW=314.20 g/mol, 6.5 mg/kg](n=6 for NO and n=6 for H_2O_2): The animal was intravenously infused with 20% D-glucose with 6.5 mg/kg BH_4 in order to maintain hyperglycemic condition at 200 mg/dl and approximately 250 μM BH_4 in blood for 3 hours. This group was designed to evaluate if addition of eNOS coupling cofactor, BH_4, can attenuate higher blood H_2O_2 and enhance blood NO levels under hyperglycemic conditions. The dose of BH_4 was chosen based on the anti-oxidant effects observed in femoral ischemia/reperfusion model (16).

4.) Hyperglycemia + BH_2 [MW=239.23 g/mol, 4 mg/kg](n=6 for NO and n=7 for H_2O_2): The animal was intravenously infused with 20% D-glucose with 4 mg/kg BH_2 in order to maintain hyperglycemic conditions at 200 mg/dl and approximately 200 μM BH_2 in blood for 3 hours. This group was designed to evaluate the real-time blood NO and H_2O_2 change during hyperglycemic conditions with BH_2, an oxidized form of BH_4 that serves as a cofactor for uncoupled eNOS. The addition of BH_2 was expected to exacerbate hyperglycemia induced blood H_2O_2/NO changes.

5.) Hyperglycemia + L-arginine [MW=210.66 g/mol, 600 mg/kg](n=5 for NO and n=6 for H_2O_2): The animal was intravenously infused with 20% D-glucose with 600 mg/kg L-arginine in order to maintain hyperglycemic conditions at 200 mg/dl and approximately 27.6 mM L-arginine in blood for 3 hours. This group was designed to evaluate if eNOS uncoupling caused by hyperglycemia may be due to the insufficient supply of the eNOS substrate, L-arginine. The addition of L-arginine was expected to attenuate hyperglycemia induced blood H_2O_2/NO changes.

Statistics

All the data in text and figures are represented as a mean ± the standard error of the mean (SEM). When comparing more than two groups, the data was analyzed by an ANOVA followed by a post hoc Student Newman Keuls test for pairwise comparison of subgroups, when the ANOVA test was significant. Probability values of <0.05 were considered to be a statistically significant result.

Results

The animals exhibited normal vital signs for all groups throughout the experimental period. In the hyperglycemic groups, some of the rats started to urinate from the 60 minute mark and maintained the urination throughout the rest of the experimental time.

Blood glucose levels in experimental groups

The blood glucose levels in every experimental group are shown in figure 5. The baseline level of blood glucose concentration ranged from 73-81 mg/dL and there was no significant difference among the groups. Infusion of saline for 180 min slightly increased blood glucose to 97±3 mg/dL (n=2) at the end of the experiment, which is the non-hyperglycemic condition.

Blood glucose levels were all around, but slightly higher than, the 200 mg/dL target level for all of the 20% glucose infusion groups. This indicates that the infusion of the 20% D-glucose groups was able to reach 200 mg/dL glucose levels. Meanwhile, the drugs (i.e. BH_4, BH_2, and L-arginine) have no impact on blood glucose. Blood glucose was increased to 184±34 mg/dL after 20 min infusion of 20% D-glucose. Thereafter, the blood glucose was maintained around 200 mg/dL (n=6), showing: 209±39 mg/dL at 40 min, 193±49 mg/dL at 60 min, 201±55 mg/dL at 80 min, 226±17 mg/dL at 100 min, 242±31 mg/dL at 120 min, 232±38 mg/dL at 140 min, 226±44 mg/dL at 160 min, and 233±12 mg/dL at 180 min of the continuous infusion. In the 20% D-glucose infusion group with BH_4, blood glucose increased to 236±26 mg/dL (n=8) after 20 min infusion. Thereafter, the blood glucose was kept around 200 mg/dL with continuous infusion.

Blood glucose was 224±17 mg/dL at 40 min, 247±17 mg/dL at 60 min, 238±16 mg/dL at 80 min, 255±19 mg/dL at 100 min, 252±22 mg/dL at 120 min, 261±8 mg/dL at 140 min, 240±12 mg/dL at 160 min, and 245±10 mg/dL at 180 min of the continuous infusion. In the 20% D-glucose infusion group with BH_2, blood glucose was raised to 178±12 mg/dL (n=8) after 20 min infusion. Thereafter, the blood glucose was kept around 200 mg/dL with continuous infusion. Blood glucose was 184±23 mg/dL at 40 min, 256±25 mg/dL at 60 min, 252±20 mg/dL at 80 min, 236±18 mg/dL at 100 min, 239±21 mg/dL at 120 min, 226±18 mg/dL at 140 min, 241±14 mg/dL at 160 min, and 253±12 mg/dL at 180 min of the continuous infusion. Lastly, in the 20% D-glucose infusion group with L-arginine, blood glucose was raised to 204±11 mg/dL (n=11) after 20 min infusion. Thereafter, the blood glucose was kept around 200 mg/dL with continuous infusion. Blood glucose was 225±28 mg/dL at 40 min, 239±22 mg/dL at 60 min, 251±24 mg/dL at 80 min, 205±10 mg/dL at 100 min, 242±24 mg/dL at 120 min, 250±20 mg/dL at 140 min, 238±14 mg/dL at 160 min, and 244±20 mg/dL at 180 min of the continuous infusion.

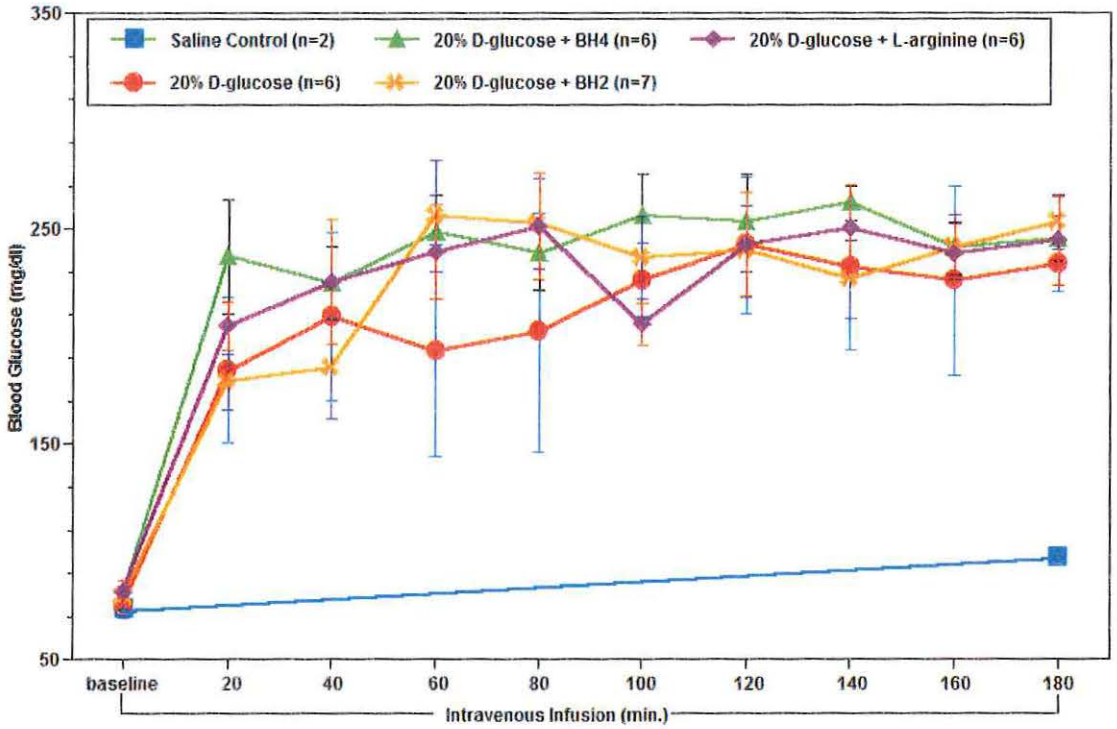

Figure 5: The comparison of blood glucose levels among saline and 20% D-glucose hyperglycemia infusion groups with or without the drugs throughout 3 hours experimental time. The saline group maintained the normal blood glucose levels. By contrast, any group containing 20% glucose increased blood glucose to about 200 mg/dL throughout the 180 minute infusion.

Blood NO levels in experimental groups

Figure 6 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 25.9±12.86 nM (n=6) at 180 minutes. In the 200 mg/dL hyperglycemic group (n=6), blood NO dropped to a significantly lower level at 100 minutes ($p<0.05$), and continuously decreased throughout the rest of infusion (all $p<0.01$) in comparison to saline group (figure 6). Blood NO levels decreased by 115.73±9.98 nM (n=6) at 180 minutes in 200 mg/dL hyperglycemia group. Furthermore, the blood NO in the 20% Glucose + BH_2 group (n=6) decreased immediately after infusion and remained significantly lower at the 40 minute ($p<0.01$), 80 minute ($p<0.05$), and from the 100 to

180 minute marks ($p<0.01$) compared to the saline group (figure 6). Blood NO levels decreased by 175.65±27.36 nM (n=6) at 180 minutes in 200 mg/dL glucose with BH_2 group. Moreover, we found that BH_2 exacerbated the hyperglycemia– induced decrease in NO levels. A significant NO decrease in 20% Glucose +BH_2 group was found at the 40 minute mark, and from the 100 through 180 minute interval compared to 200 mg/dL hyperglycemia ($p<0.05$, figure 6).

By contrast, the 200 mg/dL hyperglycemia with coupled eNOS cofactor, BH_4, or the coupled eNOS substrate, L-arginine, showed no significant difference from the saline control. Furthermore, BH_4 or L-arginine treatments also significantly increased blood NO levels compared to the hyperglycemia group. At 120 minutes, NO levels in the 200 mg/dL hyperglycemia with BH_4 only decreased by 35.8±10.01nM (n=6). Similarly, NO levels in the 200 mg/dL hyperglycemia with L-arginine only decreased by 23.33±9.12nM (n=5). This indicated that both the BH_4 and L-arginine effectively attenuated the hyperglycemia induced reduction in NO levels (figure 6).

Blood NO levels also were expressed as relative change to the saline group and illustrated in figure 7. The saline control was set as zero at each time point (figure 7). Similarly, as illustrated in figure 6, hyperglycemia significantly decreased blood NO levels. Moreover, the addition of BH_2 temporally caused significantly decreased blood NO levels at the 40 and 100 through 180 minute time intervals compared to the hyperglycemia group (figure 7). However, the addition of BH_4 or L-arginine significantly increased blood NO levels compared to the hyperglycemia group (figure 7). Both traces were getting close to the saline group.

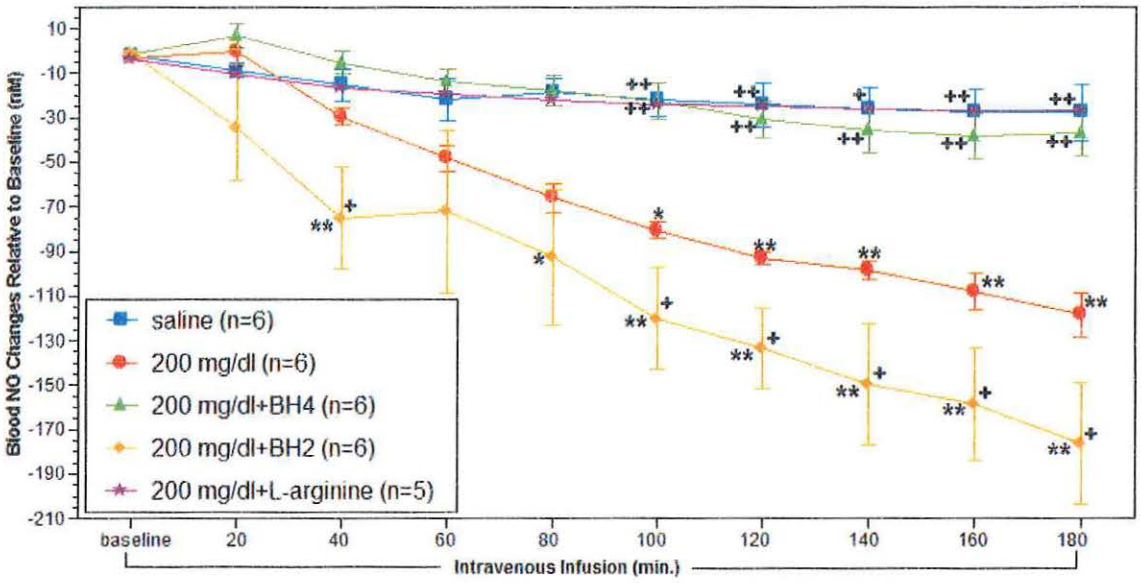

Figure 6: The comparison of blood NO levels relative to baseline among saline, 20% D-glucose, 20% D-glucose with BH_4, 20% D-glucose with BH_2, and 20% D-glucose with L-arginine (*p<0.05, **p<0.01 vs Saline, + p<0.05, ++ p<0.01 vs 200 mg/dl).

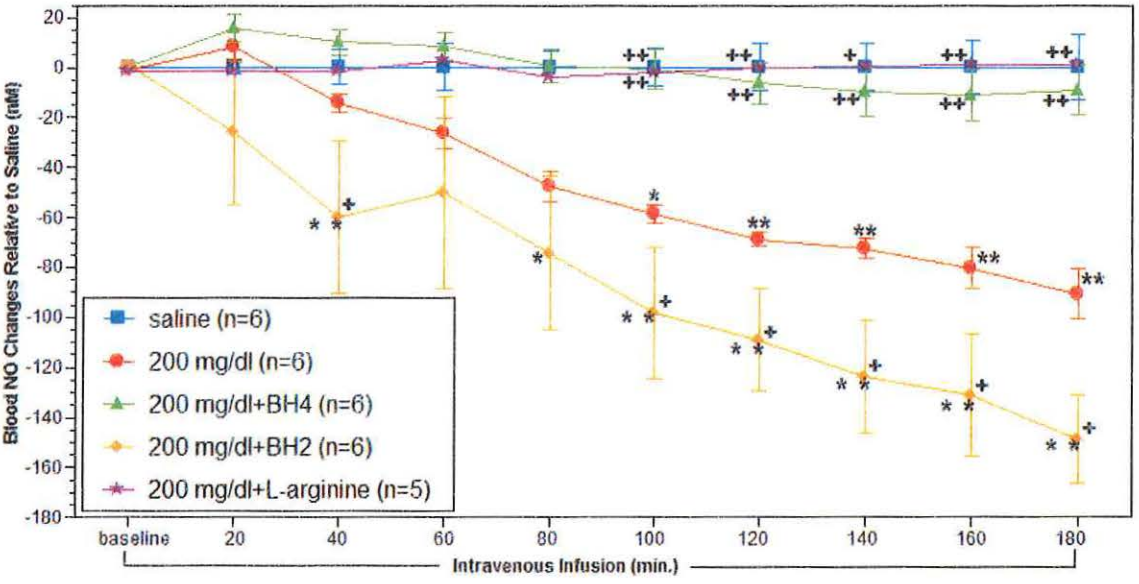

Figure 7: The comparison of blood NO levels relative to saline group among 20% D-glucose, 20% D-glucose with BH_4, 20% D-glucose with BH_2, and 20% D-glucose with L-arginine (*p<0.05, **p<0.01 vs Saline, + p<0.05, ++ p<0.01 vs 200 mg/dl).

Blood H_2O_2 levels in experimental groups

Figure 8 illustrates the blood H_2O_2 levels relative to baseline among the different experimental groups. In the saline group, blood H_2O_2 levels decreased continuously by 4.59±0.33 μM (n=7) at 180 min. In the 200 mg/dL hyperglycemic group (n=6), blood H_2O_2 remained at a significantly higher level from 40 minutes ($p<0.05$) to 180 minutes ($p<0.01$ at 60, 80, 100, 120, 140, 160, 180 min.) of infusion compared to the saline group (figure 8). At 180 minutes, blood H_2O_2 in the hyperglycemia group decreased only by 0.8±0.43 μM compared to baseline, which is significantly higher compared to the saline control ($p<0.01$).

Furthermore, the blood H_2O_2 in the 200 mg/dL hyperglycemic with BH_2 group (n=7) also remained significantly higher from the 20 minute to 180 minute intervals ($p<0.01$) compared to the saline group, but similar to the hyperglycemic group (figure 8). At 180 minutes, blood H_2O_2 in the BH_2 treated hyperglycemia group decreased only by 0.94±0.27 μM compared to baseline, which was significantly higher compared to saline control ($p<0.01$). Additionally, we found that 200 mg/dL hyperglycemia with BH_2 temporarily exacerbated hyperglycemia– induced increase in H_2O_2 levels only from 40 minutes to 60 minutes compared to 200 mg/dL hyperglycemia ($p<0.05$, figure 8).

By contrast, the blood H_2O_2 levels in 200 mg/dL hyperglycemic with coupled eNOS cofactor BH_4 only showed a significant difference from the saline control at the 20 minute interval ($p<0.01$). Blood H_2O_2 concentration continuously dropped and decreased by 3.89±0.53μM (n=6) after 180 minutes of infusion. Similarly, the blood H_2O_2 levels in 200 mg/dL hyperglycemia with L-arginine decreased by 3.31±0.043μM (n=6) after 180 minutes of infusion, which was not significantly different from the saline control or 20%

glucose + BH_4 groups. Moreover, BH_4 and L-arginine treatments significantly reduced blood H_2O_2 levels compared to those in the hyperglycemia groups. This indicates that both the BH_4 and L-arginine effectively attenuated the hyperglycemia-induced increase in H_2O_2 levels (figure 8).

In figure 9 the graph was remade to show the time course of H_2O_2 change during hyperglycemia with or without the drugs relative to saline. This is a more comprehensive illustration of the relative increase in H_2O_2 induced by the hyperglycemic condition compared to the saline group. The saline control was set as zero at each point and the differences of blood H_2O_2 levels between hyperglycemia and saline were plotted on the graph (figure 9). Similarly as illustrated in figure 8, hyperglycemia significantly increased blood H_2O_2 levels. Moreover, the addition of BH_2 temporally significantly increased blood H_2O_2 levels at the 40 and 60 minutes compared to the hyperglycemia group (200 mg/dl). However, the addition of BH_4 or L-arginine significantly reduced blood H_2O_2 levels compared to the hyperglycemia-group.

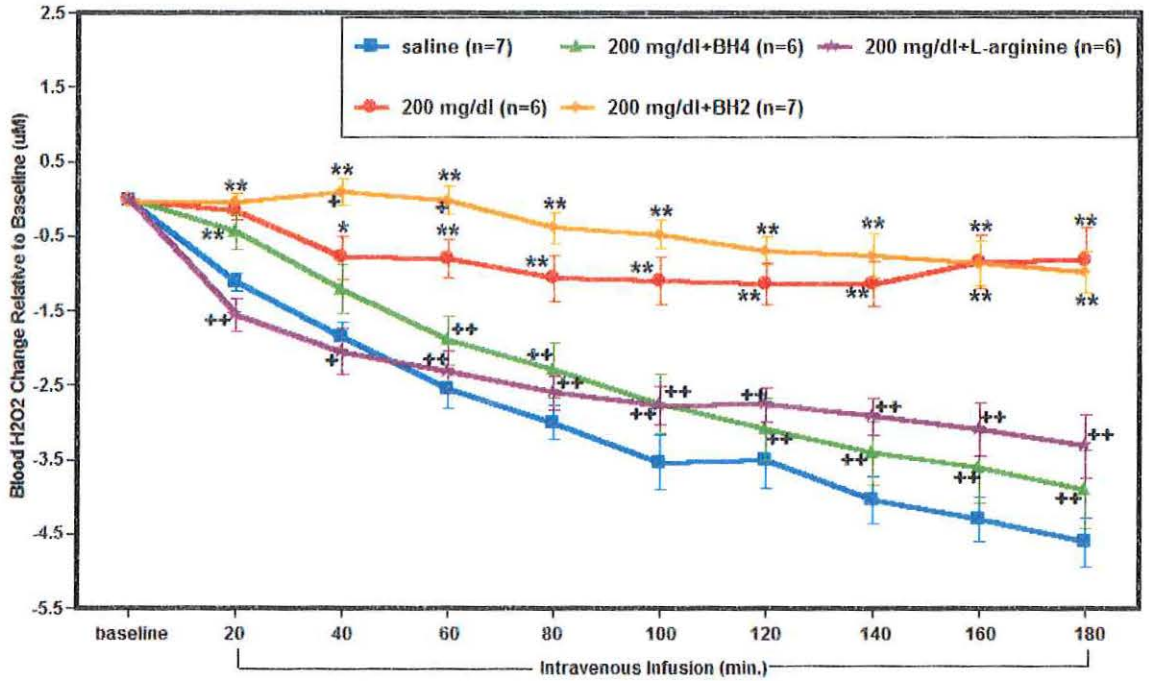

Figure 8: The comparison of blood H_2O_2 levels relative to baseline among saline, 20% D-glucose, 20% D-glucose with BH_4, 20% D-glucose with BH_2, and 20% D-glucose with L-arginine (*p<0.05, **p<0.01 vs Saline, + p<0.05, ++ p<0.01 vs 200 mg/dl).

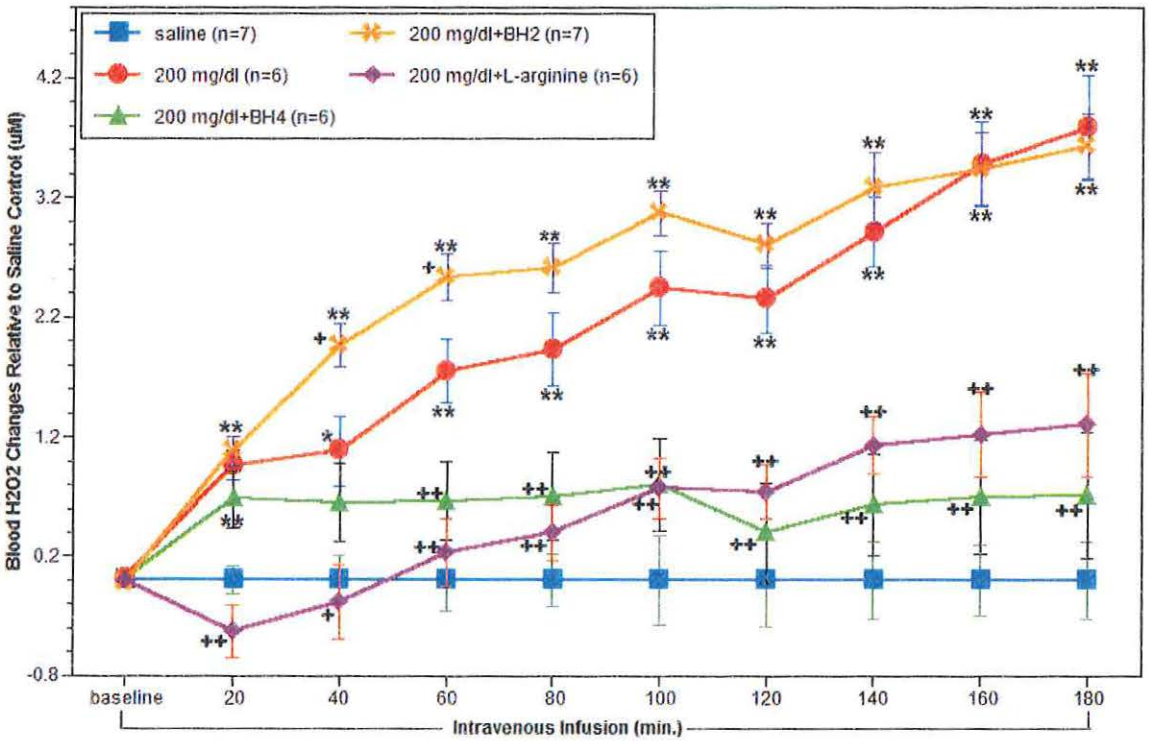

Figure 9: The comparison of blood H_2O_2 levels relative to saline among 20% D-glucose, 20% D-glucose with BH_4, 20% D-glucose with BH_2, and 20% D-glucose with L-arginine (*p<0.05, **p<0.01 vs Saline, + p<0.05, ++ p<0.01 vs 200 mg/dl).

Discussion

Summary of Results

The major findings of this study were: first, intravenous infusion of 20% of D-glucose induced 200 mg/dL hyperglycemia and the degree of the hyperglycemic condition was maintained for 180 minutes with continuous infusion. Moreover, addition of BH_4, BH_2, or L-arginine had no impact on blood glucose levels. Secondly, blood NO levels were significantly reduced during hyperglycemic conditions starting at 100 min through the rest of the hyperglycemic period as compared to the saline control group. Thirdly, under hyperglycemic conditions, blood H_2O_2 levels were significantly higher as compared to the saline control group. The fourth major finding was that 20% D-glucose with BH_2 significantly exacerbated blood NO reduction and higher blood H_2O_2 levels compared to the 20% D-glucose infusion hyperglycemic conditions. The next finding, by contrast, showed that the addition of BH_4 with hyperglycemia significantly improved blood NO levels and reduced blood H_2O_2 levels under hyperglycemia, similar to the levels found in the saline control. Finally, L-arginine with hyperglycemia significantly improved blood NO levels and reduced blood H_2O_2 levels under hyperglycemia, similar to the levels found in the saline control.

Acute Hyperglycemic rat model

We have established an acute hyperglycemia induced vascular endothelial 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. as well as a previous study performed in our laboratory by Minni (61). They showed that an initial

bolus injection of 20% D-glucose produced a maximum acute increase in blood glucose level (about 200 mg/dL). This increase was sustained when the rats received a constant glucose infusion throughout the experimental period (69). Similarly, in our hyperglycemic rats the bolus infusion of 1 ml D-glucose solution within a 4 minute time period was used for a loading dose, following which the infusion rate was reduced ten-fold to sustain the target blood glucose concentration during the 180 minute experiment infusion time. We found that this infusion method can reach 200 mg/dL hyperglycemic conditions around 20-60 min and could stay reasonably constant throughout the rest of experiment, if manipulated properly. All of the animals in all of the groups maintained relatively stable respiratory rates throughout the study conditions. Under hyperglycemic conditions with or without drugs, rats urinated at 60 minutes to the end of the infusion, which is consistent with our previous findings by Minni et al. The urination was most likely caused by hyperosmolarity in the hyperglycemic rats (61).

Mechanisms related to hyperglycemia-induced blood NO and H_2O_2 changes

Overview

Oxidative stress is a key factor in hyperglycemia-induced vascular endothelial dysfunction. The present study, in which blood NO and H_2O_2 levels were measured, confirmed Minni et al.'s finding that oxidative stress exists during acute hyperglycemia. It was previously suggested that the sources of oxidative stress during hyperglycemia include: uncoupled eNOS, NADPH oxidase, and the mitochondrial respiratory chain (9; 26; 27). It was additionally suggested that all three sources can produce excess SO in a hyperglycemic state. As a result, SO will directly quench NO to form $ONOO^-$ and reduce

NO bioavailability. Superoxide dismutase (SOD) can also further convert SO to H_2O_2. Both $ONOO^-$ and H_2O_2 can subsequently lead to eNOS uncoupling. Uncoupled eNOS produces SO instead of NO to further induce oxidative stress. Hence, the more SO being produced can in turn uncouple more eNOS. This interaction between NO and oxidative stress may well facilitate a vicious and detrimental cycle (see figure 10) which can promote vascular endothelial dysfunction, persistent inflammation, and even tissue or organ damage.

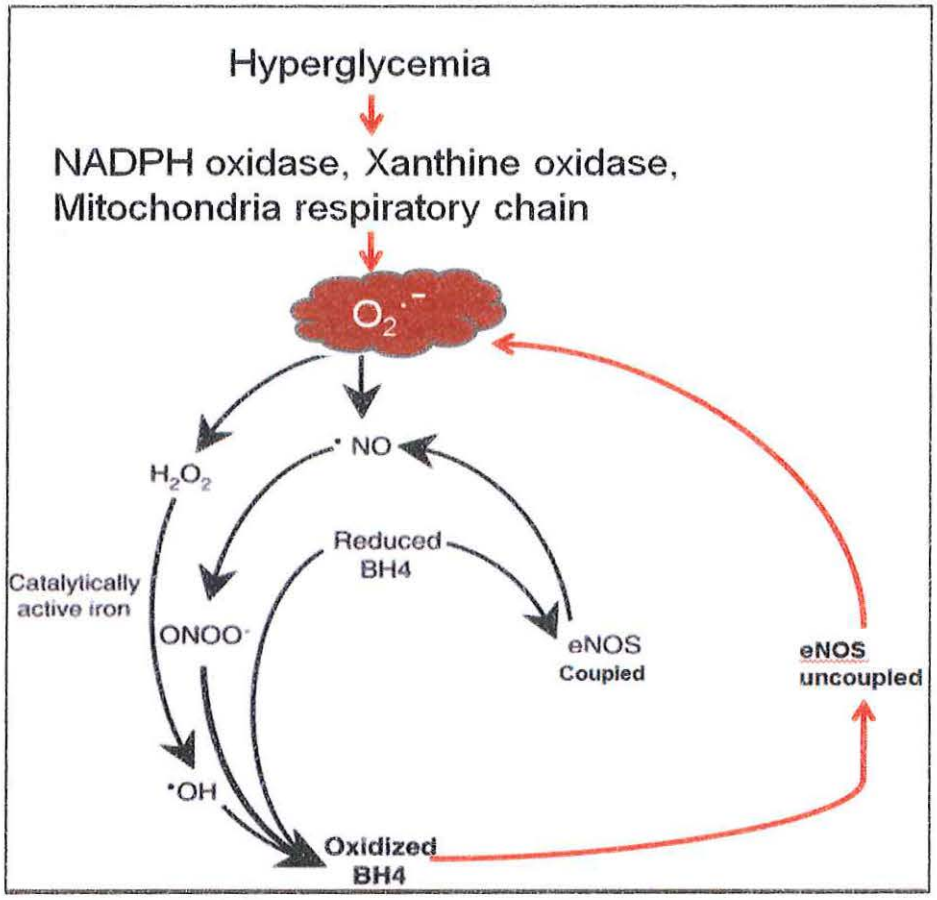

Figure 10. A representation of the vicious cycle oxidative stress during hyperglycemia conditions. An overview of the method of how the production of free radicals from hyperglycemia can propagate a continuous production of more free radicals (Modified from Wood et al., 2006).

Levels of Blood NO under Control and Acute Hyperglycemic Conditions

Postprandial hyperglycemia has been highlighted in healthy non-diabetic subjects as it has been shown to induce vascular endothelial dysfunction (13). Kawano et al. found that the post-prandial hyperglycemia after an oral glucose loading (i.e. OGTT) rapidly suppresses endothelium-dependent vasodilation, possibly by an increased production of oxygen-derived free radicals, in a group of healthy subjects (40). Title et al. found that an OGTT in 10 healthy individuals led to an acute transient attenuation of the endothelium-dependent flow-mediated dilation of the brachial artery (FMD). The method of FMD has been shown to be dependent on the release of endothelium derived NO. Consequently, when there is a decrease in the FMD, it would imply a loss of NO bioavailability and vascular endothelial dysfunction (79).

In the present study, acute hyperglycemia induced vascular endothelial dysfunction was demonstrated by measuring blood NO bioavailability in real time. Blood NO levels in the saline control group were relatively stable and dropped only slightly (approximately 26 nM relative to the baseline) within the 180 minute infusion period. The stability of blood NO in anesthetized rats had been consistently observed in the other two anesthetized animal models in our laboratory's femoral ischemia/reperfusion injury, extracorporeal shock wave lithotripsy (ESWL) animal models (16). In contrast to saline control, 200 mg/dL hyperglycemia showed to significantly decrease blood NO relative to the baseline, and the blood NO levels continuously reduced throughout the rest of hyperglycemia (200 mg/dL). At 180 min, blood NO is reduced by 115.73±9.98 nM (n=6). These findings are consistent with the clinical acute hyperglycemia induced decrease in FMD. Our data support that real time measurements of blood NO levels is a

clear indication that acute hyperglycemia significantly reduces endothelial-derived NO bioavailability and the effects can be sustained throughout the hyperglycemia.

Levels of H_2O_2 under Control and Acute Hyperglycemic Conditions

Oxidative stress is a major causal factor that has been linked to hyperglycemic induced vascular endothelial dysfunction. In a study by Marfella et al., nitrotyrosine, a marker for oxidative stress, rose significantly in 20 healthy subjects under the effects of acute hyperglycemic conditions (55). Nitrotyrosine has been found to be a suitable marker of $ONOO^-$ and nitrosative stress generation, and increased nitrotyrosine plasma levels have been found in the plasma of diabetics (15). This present study utilizes blood H_2O_2 levels to indicate SO production under acute hyperglycemia. SO has a very short half-life of seconds and it can be quickly converted into H_2O_2 by SOD. Increased blood H_2O_2 is correlated to oxidative stress under different pathophysiological conditions, such as ischemia-reperfusion, ESWL, and acute hyperglycemia (16; 61). In this study, H_2O_2 levels dropped approximately 4.50 ± 0.33 μM within 180 minutes in the saline group. This observation was presumably attributed to a decrease in body temperature when the anesthetized rat was positioned on a thermostatic platform for over 240 minutes (61). This H_2O_2 blood level decrease was also observed in our laboratory femoral ischemia/reperfusion injury and ESWL rat models (16). The 200 mg/dL hyperglycemia group had a significantly higher blood H_2O_2 level compared to saline control, dropping 0.8 ± 0.43 μM from baseline within 180 minutes. The significant increase in H_2O_2 levels started at the beginning of the intravenous infusion of 20% D-glucose (i.e. 40 min). Moreover, blood H_2O_2 levels also continuously increased when hyperglycemia infusion

was maintained, which indicates that hyperglycemia may directly induce oxidative stress via amplification mechanisms (see Fig. 10). We also proved that there is an inverse relationship between blood NO and oxidative stress (i.e. H_2O_2 levels) in blood under hyperglycemic conditions.

Coupled/Uncoupled eNOS

Normally eNOS maintains a coupled status by linking electron transfer with oxidation of L-arginine to generate L-citruline and NO in the presence of an essential co-factor BH_4 (72). BH_4 has been shown to stabilize eNOS dimer structure which has a catalytic function (26). However, under oxidative stress, BH_4 can be oxidized to BH_2 which can then occupy the eNOS oxygenase domain and block electron transfer to L-arginine. At this point, eNOS becomes uncoupled and starts to generate SO instead of NO (see figure 1) (83). Fonseca et al. found that H_2O_2 can induce a dimer collapse in the eNOS as well as a loss of a zinc from the enzyme, and both cause eNOS uncoupling (26). Additionally, the overproduced SO during hyperglycemia can bind to NO very quickly to form $ONOO^-$. $ONOO^-$ can oxidize BH_4 to BH_2 resulting in a decreased BH_4 to BH_2 ratio. Since eNOS has an equal affinity for binding to BH_4 and BH_2 it becomes uncoupled and produces SO instead of NO (17). This may be one way in which oxidative stress during hyperglycemia can propagate a vicious cycle which increases SO production and directly reduces NO bioavailability by forming $ONOO^-$. Subsequently, $ONOO^-$ and H_2O_2 further cause eNOS uncoupling to produce SO instead of NO. Thereafter, more SO is generated, while less NO is produced. This cycle can keep repeating and the ROS produced can cause serious damage to vascular endothelial function. In our present study, 200 mg/dL

with BH_2 exhibited significant blood NO reduction and higher blood H_2O_2 levels compared to the saline control conditions. Moreover, 200 mg/dL glucose with BH_2 exacerbated a significant blood NO reduction and higher blood H_2O_2 levels compared to the 20% D-glucose infusion induced hyperglycemic conditions. This indicates that adding BH_2 may further lower the BH_4 to BH_2 ratio, thus facilitating more eNOS uncoupling than hyperglycemia alone.

By contrast, 200 mg/dL glucose with BH_4 showed the opposite effects on blood NO levels compared to that in 200 mg/dL + BH_2 groups: an increase in NO that was similar to the saline control levels. Furthermore, the addition of BH_4 also showed a significant decrease in H_2O_2 compared to the blood H_2O_2 levels during hyperglycemia. Moreover, the H_2O_2 levels in BH_4 treated hyperglycemia also were similar to the saline control levels. Therefore, the addition of BH_4 effectively attenuated hyperglycemia induced vascular dysfunction and oxidative stress. These results are consistent with clinical studies that suggest that adding BH_4 can reverse OGTT-induced vascular dilatory dysfunction in normal subjects (81). A possible explanation for the effects of BH_4 is that the addition of BH_4 can increase the BH_4 to BH_2 ratio, which effectively leads to the promotion of eNOS coupling and stabilization of the dimer structure of eNOS in hyperglycemia. Therefore, uncoupled eNOS following oxidative stress induced by hyperglycemia seems to be a major factor in the exacerbation of blood NO and H_2O_2 changes and vascular endothelial dysfunction. By contrast, the promotion of coupling eNOS by adding BH_4 may well be a way to overcome these negative effects of acute hyperglycemia on the vasculature.

This present study established the roles of eNOS coupling and uncoupling on acute hyperglycemia-induced vascular dysfunction and oxidative stress by measuring blood NO and H_2O_2 levels in real-time. The acute hyperglycemia groups had significantly decreased blood NO levels and significantly higher blood H_2O_2 levels. The H_2O_2 increase occurred directly after induction of the acute hyperglycemia, whereas the reduction of endothelial-derived NO bioavailability took place from the 100 minute point of acute hyperglycemia onward. This indicates that oxidative stress is a predecessor of vascular endothelial dysfunction under the acute hyperglycemic condition, and suggests that acute hyperglycemia for a duration of 180 mins increased oxidative stress and reduced endothelial-derived NO bioavailability. We also established that adding BH_2 and inducing more eNOS uncoupling can further decrease NO bioavailability and increase blood H_2O_2. Furthermore, BH_4 may attenuate the levels of blood NO and H_2O_2 to levels similar to those found in saline control samples. These results indicate that most acute hyperglycemic changes can be attenuated by promoting eNOS coupling.

L-arginine

L-arginine serves as the substrate in the process of coupled eNOS for producing NO and has been shown to stabilize the eNOS dimer structure, which has catalytic function, in a mechanism similar to BH_4 (26). This is an important factor as insufficient L-arginine can simulate the enzymatically uncoupled molecular oxygen to produce SO, resulting in SO production instead of NO. Mah et al. has shown that plasma L-arginine decreases in a postprandial period of acute hyperglycemia (53). Endothelial cells express arginases, of which arginase II is the predominant isoform, whose function is to metabolize L-arginine

into L-ornithine and urea (24). Mah et al. proposes that an upregulation of arginase may be responsible for the decrease in arginine (53). It has been shown that there is a greater upregulation of arginase activity in diabetic individuals compared to healthy controls. The study by Mah et al. also gives us novel evidence that acute hyperglycemia in healthy young participants increases the asymmetric dimethyl-L-arginine (ADMA):arginine ratio, an index of reduced NO biosysnthesis. ADMA is an endogenously produced competitive inhibitor of eNOS, which competes locally with L-arginine. Oxidative stress caused by the acute hyperglycemia may also inhibit dimethylarginine dimethylaminohydrolase (DDAH)-mediated ADMA degradation, which results in a greater intercellular ADMA concentration (53). Mah et al. concludes that the increase in ADMA:arginine during acute hyperglycemia may cause impairments in the eNOS-mediated biosysnthesis of NO and vascular function. L-arginine may also have non-substrate effects, such as a potential direct radical scavenging effect based on the guanidine nitrogen group (27). In this current study, 200 mg/dL with L-arginine produced effects similar to those produced by BH_4. The addition of L-arginine led to an increase in NO levels compared to NO levels during hyperglycemia, which were similar to the saline control levels. In contrast, addition of L-arginine showed a significant decrease in H_2O_2 compared to H_2O_2 levels during the hyperglycemia which is also similar to the saline control levels. Therefore the L-arginine also effectively attenuated the hyperglycemia induced vascular dysfunction. These results suggest that an increase in the substrate (i.e. L-arginine) of coupled eNOS may maintain coupled eNOS function, thus continuously producing NO. We have also noticed that L-arginine had an initially quicker protective function compared to BH_4 from

the oxidative stress cause by hyperglycemia, which may be attributed to antioxidant properties of L-arginine.

Other Possible Sources of ROS Production

NADPH Oxidase

NADPH oxidase is a multi-subunit enzyme that catalyzes SO production by the reduction of molecular oxygen by using NADPH as an electron donor. In addition to neutrophils, where it was originally discovered, NADPH oxidase is also present in endothelial cells, vascular smooth muscle cells, fibroblasts, and some other cells (87). The physiological nonphagocytic NADPH oxidase derived SO is usually in low concentrations and has been implicated in the regulation of migration, activation, proliferation, vascular tone, and vascular cell growth (10; 80). However, if NADPH oxidase is upregulated the increased quantity of SO produced can result in oxidative stress. Many studies have indicated that hyperglycemia can activate the enzyme PKC in vascular endothelial cells. Hyperglycemia may stimulate SO production through a PKC-dependant activation of NADPH oxidase in vascular endothelial cells (38). Inogouchi et al. found that exposure of aortic endothelial cells to a hyperglycemic level of 400 mg/dL for 72 hrs led to a significant increase in free radical production compared with cells exposed only to a normal glucose level of 100 mg/dL (38). Inogouchi also found that the increase in free radical production was attenuated with the use of a NADPH oxidase inhibitor, diphenylene iodonium (38). Inoguchi's results suggest that hyperglycemia may induce ROS production by vascular NADPH oxidase following PKC activation. In addition, it has been found that SO production is significantly increased in streptozotocin-

induced diabetic rats two weeks after the onset of diabetes. Sonta et al. found that a NADPH oxidase inhibitor or PKC inhibitor can significantly attenuate this oxidative stress in these streptozotocin-induced diabetic rats (74). Moreover, the transition of coupled eNOS to uncoupled eNOS demands oxidative stress. Overproduction of SO via activated NADPH oxidase may be a major source of oxidative stress to further cause eNOS uncoupling. All of these findings makes the investigation of a NADPH oxidase inhibitor to attenuate the blood H_2O_2 increase/NO decrease in our acute hyperglycemia model an exciting field for future studies.

Mitochondrial SO production

Human mitochondria produce the majority of the body's required adenosine triphosphate through the process of oxidative phosphorylation (21). The system of oxidative phosphorylation includes five large multienzyme complexes called electron transfer chain (ETC) complex I-IV, and the ATP synthase complex V (32). Under normal physiological circumstances, in most tissues, this system is an important source of ROS (21). Meanwhile, SOD inside mitochondria removes SO to protect the mitochondria from oxidative injury (54). During hyperglycemia, it has been hypothesized that tricarboxylic acid (TCA) cycle electron donors (NADH and $FADH_2$) become increased, which in turn increases the mitochondrial membrane potential. Once mitochondrial membrane potential reaches a critical point, electron transport at complex III is inhibited, further promoting the half-life of free-radical intermediates of co-enzyme Q, which lead to the reduction of molecular oxygen to SO (9). Srinivasan et al. found that chronic exposure of human aortic endothelial cells to elevated glucose (25 mM)

reduced total nitrite levels by 46%, eNOS mRNA by 46%, and eNOS protein by 65%. This effect was attenuated by the specific inhibition of ROS production through the mitochondrial electron transport chain (ETC) (75). These findings demonstrate that mitochondrial derived SO are another possible source of reducing NO bioavailability under hyperglycemic conditions. This mechanism mediating eNOS uncoupling should be further evaluated under acute hyperglycemia in vivo, as well by measuring blood NO and H_2O_2.

D-glucose scavenging of NO

Glucose alone, in vitro, has been shown to promote NO loss. Brodsky et al. found that glucose has a direct scavenging effect by quenching NO (8). Brodsky et al. provided experimental evidence suggesting that when blood glucose was elevated to 270 mg/dL in healthy human subjects, there was a significant reduction in NO-mediated vasoactivity which lead to an increase in systolic and diastolic blood pressure and a decrease in blood flow to the leg (8). This may well serve as another possible mechanism to explain the drop in blood NO levels under acute hyperglycemic conditions.

Limitations

Most of the information about hyperglycemia's effects on vascular endothelial dysfunction has been derived from investigations that utilized a diabetic model. The acute hyperglycemia employed in the study reported here was induced in a normal, healthy rat model with normal insulin secretion that responded to a blood glucose change. Insulin has been shown to have vasoactive properties that can cause less SO and more NO production. However, Williams et al., showed that the effects of acute hyperglycemia on

endothelium-dependent vasodilation were comparable after using octreotide to inhibit pancreatic secretion of insulin, suggesting that hyperglycemia is the likely offender (86). Moreover, since there was a continuous infusion of D-glucose in the present study, the excessive amount of glucose overrode the effects of insulin to maintain the acute hyperglycemic conditions. Therefore, the blood NO and H_2O_2 level changes seen in this study were indicative of the negative effects of acute hyperglycemia on vascular function.

Future Studies

Upon completion of this study, samples of blood, heart, aorta, and kidney were collected for future analysis. Plasma will be used to conduct a fluorescence Griess Assay to measure the blood nitrite levels to confirm the NO data. Furthermore, blood malondialdehyde concentration could be measured to reflect systemic lipid oxidation in order to confirm systemic oxidative stress induced by the acute hyperglycemic conditions. Plasma BH_4 and BH_2 ratios could also be measured by high-performance liquid chromatography (HPLC) to determine if there was legitimate eNOS coupling or uncoupling among different experimental conditions. Another way to look at the eNOS coupled vs uncoupled ratio would be to do a western blot analysis by measuring eNOS dimer and monomer levels. ADMA and L-arginine also could be measured to understand the importance of acute hyperglycemia on the ADMA:arginine ratio and to compare with Mah's studies.

Furthermore, this is a study to measure blood NO and H_2O_2 changes in real-time as a means to establish the acute hyperglycemic effects on vascular endothelial function and oxidative stress. We found that acute hyperglycemia groups had a significant drop in

blood NO levels and a significantly higher blood H_2O_2 level in comparison to the saline control group. We also established that BH_4 and L-arginine attenuated this blood NO and H_2O_2 change under hyperglycemia whilst BH_2 exacerbated it. Our data suggests that eNOS coupling and uncoupling status is a major contributor to vascular endothelial normal and abnormal function. Moreover, NADPH oxidase and mitochondrial SO production may be the major sources causing eNOS uncoupling under hyperglycemic conditions. Therefore, further studies are needed to explore these related mechanisms by using NADPH oxidase inhibitor (diphenylene iodonium, gp91ds-tat) or mitochondrial-targeted antioxidant (i.e. SS-31 or mitoquinone).

Conclusion

In summary, we found that acute hyperglycemia significantly induces oxidative stress and vascular endothelial dysfunction. Moreover, the addition of BH_2, which facilitates eNOS uncoupling, exacerbates both changes under hyperglycemia. By contrast, the addition of L-arginine, or BH_4, which promote eNOS coupling, significantly improves vascular endothelial function and reduces oxidative stress. Therefore, this study provides novel evidence for the role of eNOS uncoupling in vascular endothelial dysfunction and oxidative stress under acute hyperglycemia.

Clinical Relevance

As acknowledged in the introduction, hyperglycemia is linked to vascular endothelial dysfunction by increasing oxidative stress. Vascular endothelial dysfunction due to this prolonged oxidative stress has been associated with cardiovascular disease and atherosclerosis. The purpose of this study was to establish the role of eNOS in vascular

endothelial dysfunction in an acute hyperglycemic model to help prevent vascular dysfunction and other circulatory complications in non-diabetic or prediabetic individuals. Hyperglycemia has been linked to the progression of diabetic nephropathy which was demonstrated when Piwkowska et al. showed that hyperglycemia produced reactive oxygen species causing morphological and functional changes in mouse podocytes (66). Maintaining normal glucose levels during surgery has been shown to be important in avoiding complications such as blood clots and stroke as well. Psychological stress is another factor that can cause hyperglycemia, so eliminating chronic psychological stress will be significant in avoiding future vascular complications also.

This present study should also bring more attention to the hazards of postprandial hyperglycemia. It has been well established that an excess of dietary saturated fat and dietary sodium consumption is directly associated with cardiovascular diseases. To live a healthy life and prevent some of these problems, individuals have learned to reduce their intake of dietary fat and sodium. However, commonly consumed foods that raise your blood glucose like soda, fruit juice, and candy are not commonly seen through the eyes of the public as hazardous to their cardiovascular health. A 20 oz bottle of one of the leading grape sodas contains 82.5 g of sugar. Consuming this amount should lead to the anticipation that vascular endothelial function changes similar to those seen in individuals in the 75 g post OGTT study mentioned earlier would occur. This study presents some evidence to raise awareness of the possible detrimental impacts of a high sugar/high glucose diet on vascular function.

Resources

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