Characterization of Acetylcholine-Mediated Vasodilation in Mourning Dove Arteries Under Normoglycemic and Hyperglycemic Conditions

by

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ABSTRACT

Birds have plasma glucose levels that are 1.5-2 times greater than mammals of similar body mass in addition to higher free fatty acid concentrations, both of which would typically impair endothelium-dependent vasodilation if observed in mammals. Endothelium-dependent vasodilation can be stimulated in mammals through the use of acetylcholine (ACh), which primarily acts through nitric oxide (NO) and cyclooxygenase (COX)-mediated pathways, with varying reliance on endothelial-derived hyperpolarizing factors (EDHFs). Very few studies have been conducted on small resistance systemic arteries from birds. The hypothesis was that because birds have naturally high glucose and free fatty acid concentrations, ACh-induced vasodilation of isolated arteries from mourning doves (*Zenaida macroura*) would be independent of endothelial-derived factors and resistant to high glucose-mediated vascular dysfunction. Small resistance mesenteric and cranial tibial (c. tibial) arteries were pre-constricted to 50% of resting inner diameter with phenyleprine then exposed to increasing doses of ACh (10^{-9} to 10^{-5} µM) or the NO donor, sodium nitroprusside (SNP; 10^{-12} to 10^{-3} µM). For both vessel beds, ACh-induced vasodilation occurred mainly through the activation of potassium channels, whereas vasodilation of mesenteric arteries additionally occurred through COX. Although arteries from both vessel beds fully dilated with exposure to sodium nitroprusside, ACh-mediated vasodilation was independent of NO.

To examine the effect of high glucose on endothelium-dependent vasodilation, ACh dose response curves were conducted following exposure of

isolated c. tibial arteries to either a control solution (20mM glucose) or high glucose (30mM). ACh-induced vasodilation was significantly impaired ($p = 0.013$) when exposed to high glucose, but normalized in subsequent vessels with pre-exposure to the superoxide dismutase mimetic tiron (10 mM). Superoxide concentrations were likewise significantly increased ($p = 0.0072$) following exposure to high glucose. These findings indicate that dove arteries do not appear to have endogenous mechanisms to counteract the deleterious effects of oxidative stress. Additional studies are required to assess whether endogenous mechanisms exist to protect avian vascular reactivity from systemic hyperglycemia.

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Chapter 1

INTRODUCTION

Endothelial dysfunction is known to be part of the early etiology for the development of atherosclerosis and cardiovascular disease. This is characterized by the endothelial homeostatic mechanisms shifting to a vasoconstrictive, pro-thrombic and pro-inflammatory condition (Potenza, Gagliardi, Nacci, Carratu', & Montagnani, 2009). Impaired endothelium-dependent vasodilation as measured using acetylcholine (ACh) can result from a variety of mechanisms. Reductions in the bioavailability of nitric oxide (NO) may occur through diminished endothelial nitric oxide synthase (eNOS) expression or activity resulting in impaired vasodilation. Moreover, an increase in superoxide ($O2^{\cdot-}$) impairs vasodilation by activating enzymes such as NADPH-dependent oxidases, and protein kinase C (PKC) which decreases NO bioavailability (Potenza et al., 2009).

Oxidative stress is exemplified by the production of reactive oxygen species (ROS) in excess of the ability to scavenge free radicals (King & Loeken, 2004). In normal concentrations glucose is metabolized via glycolysis to generate pyruvate, which goes through the tricarboxylic acid cycle to produce NADH and $FADH_2$. These molecules then pass through the mitochondrial electron transport chain to finally produce ATP that is used to supply tissues with energy. When glucose exceeds the amount that can be normally processed, the electron transport chain becomes saturated, and the production of ROS is increased. Namely, hydrogen peroxide (H_2O_2) and $O_2^{\cdot-}$ are two free radicals that may alter vasodilation. Superoxide in particular may combine with NO to produce the ROS

peroxynitrite ($ONOO^-$) thereby reducing NO bioavailability resulting in impaired vasodilation (West, 2000). H_2O_2, having vasoactive properties, may exert direct effects on vasodilation (Jerca, Jerca, Gabriela, Constantinescu, & Lupusoru, 2002). Acute and prolonged hyperglycemia have been shown to cause oxidative stress which, in turn, leads to endothelial dysfunction through enhanced $O_2^{\cdot -}$-mediated scavenging of NO despite normal production of NO through eNOS (Jin & Loscalzo, 2010). Both NO-dependent and independent endogenous vasodilatory pathways are inhibited by hyperglycemia within the vasculature (Potenza et al., 2009). When NO is not available in proper concentrations, arteries are more constricted leading to increased blood pressure. Many pathways affected by hyperglycemia ultimately increase oxidative stress, and many of these metabolic pathways increase activation of PKC. Increased PKC may, in turn, activate mitochondrial NADPH oxidase leading to further increases in ROS production (King & Loeken, 2004).

Acetylcholine is an endothelium-dependent vasodilator as its effects are dependent on the presence of the endothelium to cause vasodilation, and it is frequently used to evaluate endothelial function (Medow, Glover, & Stewart, 2008). Binding of ACh to its receptor on the endothelial layer of blood vessels activates two main pathways to elicit vasodilation; one dependent on endothelial derived relaxing factors (EDRF) such as NO and cyclooxygenase (COX) as well as another dependent on endothelial derived hyperpolarizing factors (EDHF) which activate potassium channels to cause potassium efflux. The effect of EDRF plays a larger role in small arteries, less than 200 μm in diameter whereas NO is

more dominant in larger arteries (de Wit & Wolfle, 2007). Through the NO-dependent pathway, ACh triggers an increase in the levels of calcium in endothelial cells, which activates eNOS to produce the vasodilator NO. Nitric oxide is produced in the cytosol of vascular endothelial cells and diffuses into nearby vascular smooth muscle cells where it increases the synthesis of 3,5-cyclic guanosine monophosphate (cGMP), and decreases cytosolic Ca^{2+} concentrations by inhibiting voltage-gated Ca^{2+} channels to ultimately produce vasorelaxation (Jin & Loscalzo, 2010). Along with this well-established mechanism for NO induced vasorelaxation, NO also produces vascular smooth muscle cell relaxation by activating a variety of potassium channel subtypes causing hyperpolarization of the cells (Edwards, Feletou, & Weston, 2010). Briefly, ACh-mediated vasodilation through COX pathways occurs through the activation of prostanoids such as PGI_2, that elicit direct vasodilation on vascular smooth muscle cells (Medow et al., 2008) In mammals these pathways are clearly characterized; however, in avian vasculature ACh-mediated vasodilation has not been well-defined.

Prior studies of isolated small resistance mesenteric arterioles from mourning doves (*Zenaida macroura*), revealed that ACh does not act through NO (Jarrett et al., In Review). My preliminary studies investigating the c. tibial muscle arterioles showed the same results. The observed NO-independent ACh-vasodilation in avian arteries suggests a role for other EDRFs, like COX, or EDHFs in mediating vasodilation in the microvasculature of birds. EDHF causes vasodilation by influencing the electrochemical gradient of cells. In vascular

smooth muscle cells, membrane hyperpolarization is caused by an efflux of K^+ resulting in smooth muscle cell relaxation and vasodilation (Jackson, 2005). This ACh-mediated activation of EDHF in rats is impaired in isolated mesenteric arteries with an acute exposure to high glucose (Ozkan & Uma, 2005). For birds, preliminary studies of endothelium-disrupted arteries also show that ACh is at least partially dependent on the endothelium in both mesenteric and c. tibial vascular beds. Endothelial disruption is accomplished by rubbing the lumen of the artery with a strand of moose mane inserted into the vessel. Endothelial cells are then flushed through the vessel before it is cannulated. This method of endothelial disruption has been performed in previous studies of rat coronary arteries (Cherng, Campen, Knuckles, Gonzalez Bosc, & Kanagy, 2009). Moreover, pilot data examining sensitivity of the vessels to a NO donor has also revealed that the vascular smooth muscle cells in the arterioles are sensitive to NO, but the exact mechanism of action is not clear. Therefore, the potential roles for COX in ACh-vasodilation and the EDHF pathway in both ACh and NO-mediated vasodilation will be examined.

The use of an avian model for characterization of ACh-dependent vasodilation is novel due to their unique ability to resist oxidative stress caused by naturally hyperglycemic states. Avian physiological plasma glucose levels are 1.5-2 times greater than that of mammals of comparable body mass (Beuchat & Chong, 1998; Braun & Sweazea, 2008). Despite this, birds appear to be remarkably resistant to the development of oxidative stress in brain, heart and kidney tissues (Ku & Sohal, 1993). From previous studies in mourning doves,

levels of ROS in isolated mesenteric arterioles using a fluorescent indicator were similar to levels found in rat mesenteric arterioles (C. L. Smith et al., 2011).

Therefore, the purpose of this proposal is to examine mechanisms that may prevent hyperglycemia-mediated endothelial dysfunction in avian arteries. Though this proposal, the pathways(s) through which ACh mediates vasodilation will be characterized and examined for their resistance to hyperglycemia-mediated oxidative damage. ACh-mediated vasodilation is examined by first pre-constricting isolated arteries to approximately 50% of their resting inner diameter and then exposing the arteries to increasing concentrations of ACh. In my preliminary studies I examined two different vasoconstrictors, phenylephrine (PE) and potassium chloride (KCl) to assess the appropriateness of each as their mechanisms of eliciting vasoconstrictions are different. Phenylephrine is a powerful vasoconstrictor and its action is mediated by its effect on α_1-andrenergic receptor on the smooth muscle cell. Binding of phenylephrine activates G_q-proteins, which then stimulates phospholipases C, A_2, and D to cause an intracellular increase of Ca^{2+} via mobilization from intracellular stores, resulting in vasoconstriction (Lippton, Armstead, Hyman, & Kadowitz, 1987). Potassium chloride causes vasoconstriction through depolarization of the smooth muscle cell membrane. Therefore, by increasing the concentration of K^+ in exchange for Na^{2+} in the buffer solutions used to bathe the vessels, sustained vasoconstriction can be obtained (Schuurman & Villamor, 2010). However, my preliminary studies demonstrate that while ACh fully reverses PE-mediated vasoconstriction, it does

not fully reverse KCl-mediated vasoconstriction. For this reason, all proposed vasodilation protocols will use PE as the vasoconstrictor.

Research Aims and Hypotheses

Hypothesis 1: Because birds have naturally high blood glucose and fatty acid concentrations, ACh-mediated vasodilation in small resistance arteries from doves is expected to be independent of NO and other endothelial-derived factors.

Specific Aim 1: To complete the characterization of EDRF and EDHF vasodilatory pathways in isolated avian mesentery and c. tibial small resistance arteries by performing ACh and SNP dose response curves in the presence of the COX inhibitor, indomethacin, and the K^+ channel inhibitor, TEA.

Specific Aim 2: Analyze plasma nitrate and nitrite levels in mourning doves.

Hypothesis 2: The high endogenous antioxidant capacity of birds protects from hyperglycemia-induced tissue damage thereby maintaining normal vascular function in *ex vivo* arteries exposed to high glucose conditions.

Specific Aim 1: Characterize the effects of acute exposure to hyperglycemic conditions on ACh-mediated vasodilation in isolated c. tibial arteries. Vascular ROS will also be measured using the fluorescent indicator DHE.

Definition of Terms

Acetylcholine (ACh): endothelium-dependent vasodilator which binds to receptors on the endothelial layer of blood vessels which induces vasodilation through EDRF and EDHF-mediated pathways.

Cyclooxygenase (COX): An enzyme that activates the synthesis of the prostaglandins, prosacyclin and thromboxane that are mediators or inflammatory reactions, vasoconstriction and vasodilation.

Endothelial Derived Hyperpolarizing Factor (EDHF): electrical signal that stimulates vasodilation by hyperpolarizing blood vessels and is generated and released by the endothelium.

Endothelial Derived Relaxing Factors (EDRF): term used to describe the release of nitric oxide and cyclooxygenase by the endothelium which elicits vasodilation of blood vessels.

Endothelial Dysfunction: pathological state of the endothelium. This can be the first step in the etiology of atherosclerosis and cardiovascular disease.

Endothelium-dependent vasodilation: term used to describe the effect of vasodilators that are dependent of the presence of the endothelial cells to elicit vasodilation.

Endothelial nitric oxide synthase (eNOS): key enzyme that generates NO in blood vessels from the endothelium. This differs from iNOS (inducible) and nNOS (neuronal).

Nitric Oxide (NO): endogenous compound that plays an important role in cellular signaling to induce vasodilation.

Nω-nitro-L-arginine (LNNA): a potent inhibitor of nitric oxide synthase, which inhibits calmodulin, a calcium dependent enzyme, to prevent the production of endothelium-mediated relaxation of vascular smooth muscle by NO.

Phenylephrine (PE): α 1-adrenergic receptor agonist that elicits vasoconstriction by increasing intracellular calcium concentrations.

Protein kinase C (PKC): a family of protein kinase enzyme isoforms that produce signal transduction cascades by phosphorylation of hydroxyl groups of amino acid residues.

Reactive Oxygen Species (ROS): chemically reactive molecules that contain oxygen and are highly reactive due to the presence of unpaired valence shell electrons. ROS are common byproducts of metabolism. A high level of ROS is known to cause oxidative stress. Typical ROS include superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$).

Sodium nitroprusside (SNP): inorganic compound which is a NO donor.

Tetraethylammonium chloride TEA: a nonspecific potassium channel blocker.

Delimitations and Limitations

There are aspects of this proposed study that limit the scope, which are conscious inclusionary or exclusionary methods, defined as delimitations. Most notably, the animal model for this study only includes wild-caught mourning doves from the ASU Tempe campus. This is advantageous over breeder-supplied birds since the animals will be living in their natural state prior to capture and therefore the data should be representative of the wild population. However, the birds will not likely be representative of the rural population or the population of mourning doves as a whole although we cannot exclude the possibility of capturing rural birds since this is a migratory species.

The studies will also be looking at the *ex vivo* effects of acute hyperglycemia versus *in vivo*. This will allow for investigation into the native protective effects against hyperglycemia without external influences when investigating the entire animal as opposed to the isolated vessel. Future studies will measure the effects of hyperglycemia *in vivo* by injecting the birds with an insulin antibody known to cause hyperglycemia, and then testing their blood flow using fluorescent microspheres.

Another delimitation of this study is associated with the specified arteries that will be examined. This study is proposing to look at mesenteric and c. tibial arteries, not arteries from vital organs. However, skeletal muscle arteries are critical for glucose delivery to muscles and mesenteric arteries are from a systemic vascular bed that is important for blood pressure regulation. Both vascular beds are well-suited for this type of application due to the ease of extraction of a suitable sized artery without damage. In preliminary studies, the mesenteric and c. tibial arteries from mourning doves had similar vasodilatory pathways (Jarrett et al., In Review). Characterizing vasodilation with ACh is another delimiting factor, because there are numerous vasodilatory drugs to choose from. Acetylcholine was chosen, because it is a well-known endothelium dependent vasodilator that has been studied in many avian and mammalian animal models.

This study has some limitations of design and methodology. For example, vascular smooth muscle calcium levels are not simultaneously measured during the concentration response curves since our lab does not currently have the

capacity for these analyses. Our lab is additionally not set up to measure potassium currents using microelectrodes to characterize the EDHF response. Instead, we are proposing to use the nonspecific potassium channel blocker TEA. If potassium is determined to be involved in ACh and/or SNP-mediated vasodilation, then the specific potassium channels involved will be determined in future studies using commercially available antagonists.

Chapter 2

REVIEW OF LITERATURE

Endothelial Dysfunction

Endothelial dysfunction is the first step in the pathogenesis of atherosclerosis and can be an undetected risk factor that precedes clinical manifestations of cardiovascular diseases (Frick & Weidinger, 2007; Potenza et al., 2009). A thin layer of cells lining the interior of blood vessel walls characterizes the endothelium. Before the 1980's the endothelium was thought to be a mere barrier between the circulation and the vascular smooth muscle cells (VSMC) lining the macro and microvasculature. It is now known that the endothelium plays a crucial role in the homeostatic mechanisms that regulate blood vessel reactivity, modulation of coagulation and inflammatory processes (Somani, Steiner, & Hebbel, 2010). Endothelium-mediated vasodilation can be assessed both directly and indirectly, which gives a proxy for interpreting endothelial dysfunction. The mechanisms that underlie this early marker in the progression of vascular dysfunction that will be discussed in this review will focus on oxidative stress and, in particular, hyperglycemic conditions that lead to this imbalance.

The role of the endothelial cells was first discovered in 1980 with *in vivo* experiments conducted on isolated rabbit aortas (Furchgott & Zawadzki, 1980). Acetylcholine was used in the experiments to relax the blood vessels. However the researchers inadvertently discovered that ACh produces marked vasoconstriction if these blood vessels have had their intimal surface disrupted by

unintentional rubbing of the inner lining of the vessel (i.e. the endothelium). It was then proposed that the endothelium produced a relaxing substance other than bradykinin and prostacyclin. The term endothelium-derived relaxation factor (EDRF) was used to describe what was later to be revealed as nitric oxide (NO) (Palmer, Ferrige, & Moncada, 1987). Currently, the term EDRF is all-encompassing of the soluble mediators of vasorelaxation that are dependent on the endothelium including: nitric oxide, bradykinin, and prostaglandins. Endothelial-derived hyperpolarizing factor (EDHF) has also since been described to play a role in vasorelaxation through calcium activated potassium channels located on the endothelial and vascular smooth muscle cells(Frick & Weidinger, 2007; Parkington, Tare, & Coleman, 2008).

Measurement of Endothelial Function

Measuring endothelial function is important for its clinical relevance of indicating cardiovascular risk in its infancy and can be performed in various ways. Assessment of its function can be performed via intracoronary ACh test, strain-gauge forearm plethysmography and flow-mediated vasodilation (FMD) of the brachial artery (Kasprzak, Klosinska, & Drozdz, 2006). The intra-arterial infusion test using ACh is moderately operator-independent and gives a direct measure of *in situ* diameter change to this potent vasoconstrictor, which is highly reflective of endothelial vasoresponsiveness. This technique is highly invasive and not appropriate for large population studies. Strain-gauge forearm plethysmography is another reliable method that involves cannulation of the

brachial artery and acetylcholine infusion to measure endothelial function. The plethysmography method allows for mechanisms of endothelial-mediated vasodilation to be assessed and is another invasive and time consuming method. The FMD method of determining endothelial function is a non-invasive method with defined procedures and guidelines for assessment of results. This technique in brief, uses an ultrasound device to measure inner arterial diameter responses to hyperemia induced by a blood pressure measuring cuff (Frick & Weidinger, 2007).

Mechanistic measurements of endothelial function can be assessed in both human and animal models to elucidate the pathophysiology behind the endothelium-mediated responses to vasodilators and conditions that impair endothelial function such as acute and chronic hyperglycemia (Klotz, Gaehtgens, & Pries, 1995; Ozkan & Uma, 2005; Williams et al., 1998). In animal models and human biopsies, isolated arteries are used with various antagonistic and agonistic drugs that depict the mechanisms of endothelium-dependent and independent vasodilation after constricting the vessel with a vasoconstrictor drug and dilating with increasing doses of a vasodilator.

Hyperglycemia and Vascular Dysfunction

High blood glucose (hyperglycemia) is the defining characteristic of diabetes and occurs when glucose is not adequately absorbed by skeletal muscle and adipose tissue due to insulin resistance, in the case of type 2 diabetes mellitus, and “impaired” insulin production for type 1 diabetes mellitus. As a result,

extracellular hyperglycemia occurs leading to endothelial dysfunction induced by oxidative stress that causes pathophysiological complications such as atherosclerosis, retinopathy and neuropathy (Potenza et al., 2009). Vascular dysfunction has been well-documented to occur through increased production of reactive oxygen species (ROS) and advanced glycation end products as well as activation of protein kinase C (PKC) through increased diacylglycerol (Brownlee, 2001). The major hypothesis behind diabetic complications from hyperglycemia arises from the destructive role of ROS, specifically hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot -}$). Production of ROS may occur from oxidative phosphorylation, nicotinamide adenine dinucleotide phosphate oxidase (NADPH), glucose auto-oxidation, and xanthine oxidase (Niedowicz & Daleke, 2005). Additionally, alterations in the polyol, hexosamine, protein kinase C (PKC) and advanced glycation end-product (AGE) pathway cause increased ROS formation (Brownlee, 2001).

The activation of PKC has a vast effect regarding the pathology of hyperglycemia cardiomyopathy. A majority of the PKC family isoforms are activated through the lipid second messenger diacylglycerol which exerts a cascade of metabolic effects. PKC activation can lead to decreased expression of endothelial nitric oxide synthase (eNOS) and increased expression of endothelin-1 (ET-1) a potent vasoconstrictor, both of which promote decreased vasodilation. This activation also leads to increased pro-inflammatory gene expression of NF-κB and increased NAD(P)H oxidases, as previously discussed, leading to ROS production (Brownlee, 2001; Potenza et al., 2009). Interestingly, inhibition of

isoform PKC-β with ruboxistaurin mesylate among patients with type 2 diabetes has not been shown to restore endothelial function using strain-gauge plethysmography of forearm resistance vessels (Beckman et al., 2010).

Advanced glycation end-product (AGEs) arise from intracellular glucose auto-oxidation to form *glyoxal*, *methylglyoxal* from fragmentation of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate and decomposition of the Amadori product resulting in *3-deoxygluconsone* (Suzuki, Koh, Mizuno, Hamaoka, & Taniguchi, 1998); these three products (in italics) react with amine groups from intracellular and extracellular proteins to form AGEs (Brownlee, 2001). The plasma proteins that are altered by the AGE precursors bind to AGE receptors on endothelial and mesangial cells as well macrophages leading to receptor-mediated production of ROS (Brownlee, 2001; Yao & Brownlee, 2010) and further increases in oxidative stress.

Vascular dysfunction in both human and animal models has been attributed to hyperglycemic conditions. This deleterious effect can occur from both chronic and acute hyperglycemia. Acute hyperglycemia among subjects with impaired glucose tolerance (IGT) and type 2 diabetes mellitus during a glucose tolerance test showed decreased endothelium-dependent vasodilation as measured by brachial artery flow-mediated vasodilation (FMD)(Kawano et al., 1999). This study indicated that the decrease in FMD from the 75g oral glucose tolerance test was partially due to an increase in oxidative stress. Levels of thiobarbituric acid reactive substances (TBARS), a marker of lipid oxidation, and nitrite/nitrate (NOx) were measured and they found the concentration of TBARS increased and

was positively correlated to glucose loading, however the nitrite/nitrate levels were not altered (Kawano et al., 1999).

An *in vivo* experiment among patients with diabetes or impaired glucose tolerance showed attenuated endothelium-mediated vasodilation from acute hyperglycemia as measured by venous occlusion plethysmography (Kim et al., 2003). This study examined 8 patients by calculating the forearm blood flow with a specified dose of ACh infusion during a fasting and hyperglycemic state. The significant attenuation of forearm blood among the subjects with diabetes and IGT observed with acute hyperglycemic conditions is of importance. Additionally, these individuals were reported to have excellent glucose control and were free of known diabetic complications (Kim et al., 2003).

Acute hyperglycemia among healthy adults has also been show to attenuate endothelium-dependent vasodilation (Mah et al., 2011; Williams et al., 1998), but to have no effect on cutaneous vascular function (Charkoudian et al., 2002). Again, brachial artery plethysmography was used to assess vascular function during a fasting state compared to measurements taken during an intra-arterial infusion of 50% dextrose for 6 hours. Among the 10 healthy adults tested, dextrose induced a significant impairment in the forearm blood flow response to methacholine-mediated vasodilation (Williams et al., 1998). Similar results were observed among 16 healthy men using FMD measurements before and after 75g oral glucose ingestion, while ingestion of 75g of fructose had no effect (Mah et al., 2011). Plasma antioxidants and oxidative stress biomarkers were measured in subjects treated with fructose. No differences were found among the plasma

vitamin E, vitamin C or total antioxidant status measured by oxygen radical absorbance capacity (ORAC), but there was an increase in ferric-reducing ability of plasma (FRAP) after the ingestion of the fructose load even though no vascular impairments from fructose were found. Malondialdehyde (MDA), a measurement is reflective of lipid peroxidation, and the increased MDA was highly correlated with increased glucose and fructose along with the association between the impaired FMD. Both plasma arginine an ADMA assess NO homeostasis and this study found a decrease in plasma arginine following both glucose and fructose loads and an increase in ADMA:arginine ratio demonstrating that acute hyperglycemia may decrease NO biosynthesis (Mah et al., 2011).

In contrast to the studies showing an effect of hyperglycemia on flow mediated vasodilation, results from 28 healthy subjects using a laser-Doppler flowmetry (LDF) to measure skin blood flow showed no vasodilatory impairment with hyperglycemia. The subjects were randomly divided into three groups each receiving varying doses of glucose by infusions to mimic a euglycemic state, mild hyperglycemia and hyperglycemia. There were no significant differences observed after 6 hours among all groups compared to baseline (Charkoudian et al., 2002).

Another experiment conducted on 5 month old type 1 model diabetic rats investigated the effect of hyperglycemia on artery vasoreactivity in precapillary arterioles (Renaudin, Michoud, Lagarde, & Wiernsperger, 1999). The diabetic and non-diabetic rat skeletal muscle precapillary arteriole diameter changes were tested via an isotonic saline infusion compared to a glucose infusion. This model

showed a complete impairment in vascular reactivity with the glucose infusion among the diabetic rats but not in the non-diabetic rats (Renaudin et al., 1999). High fat diets also induce hyperglycemia and impaired vascular reactivity in rats. Animals that were fed a high fat diet showed an increase in plasma TBARS and impaired vasodilatory responses to ACh in small resistance mesenteric arteries that was attributed to increases in oxidative stress (K. L. Sweazea, Lekic, & Walker, 2010).

It is clear that in humans, chronic hyperglycemia leading to oxidative stress causes vascular dysfunction and that acute hyperglycemia in humans and animal models results in impaired vasoreactivity with indications that it is due to ROS-mediated effects on vascular function. Nevertheless, it is important to note that hyperinsulinemia associated with diabetes can have deleterious effects beyond the effect of elevated glucose. As a vasodilator, the glucose-lowering hormone insulin has also been demonstrated to affect vasoreactivity and has been shown to diminish the effect of vasoconstrictor agents (Baron, 2002). Additionally, elevated circulating free fatty acids (FFA) are associated with insulin resistance and elevated FFA levels can also cause vascular dysfunction (Baron, 2002).

Insulin also plays a role in the endothelial production of NO. Insulin signaling in the vascular endothelium can activate the gene expression of eNOS. Insulin binding to its receptor on endothelial cells can lead to two different cascade of reactions resulting in the production of NO or endothelin-1 (Baron, 2002). These two pathways show the ability of insulin to be pro-vasodilatory or

pro-constrictive. Insulin can also affect vascular smooth muscle cell contractility. NO production in the VSMC can be increased due to the effect of insulin on the upregulation of cGMP causing increased activation of eNOS. However, insulin may also activate calcium dependent potassium channels in the smooth muscle cells, which would decrease vascular tone (Baron, 2002). The dueling effect that insulin has on vasoconstriction, via increased endothelin-1 and vasodilation with the production of NO is crucial. Therefore, the homeostatic imbalance of insulin characteristic of diabetes plays an important role in vascular health.

Avian animal model

Birds have plasma glucose concentrations that are higher in comparison to mammals of similar body mass (Braun & Sweazea, 2008). This analysis comes from a data set including 162 mammalian species of varying body masses compared to 97 species of birds. The y-intercepts of P_{Glu} for the graphed data showed a negative relationship between plasma glucose and body mass for both birds and mammals and were significantly different ($p<0.001$). Additionally, prior studies that included data sets with fewer species found similar results when comparing plasma glucose and body mass among mammalian and avian species (Beuchat & Chong, 1998; Umminger, 1975). As a simple illustration, a house mouse (*Mas domesticus*) has a body mass similar to the canary (*Serinus canaria*), 20g and 22g average body mass respectively, while the maximum reported longevity is 6-fold greater among the canary(Holmes, Fluckiger, & Austad, 2001). It has been proposed that birds live approximately 3 times as long as average

mammals of similar body mass. Furthermore, the higher metabolic rate of birds that should potentially lead to an increased production of ROS in addition to body temperatures that are 3-4ºC higher than mammals does not seem to affect the longevity of this species (Austad, 2011). The implication of higher avian plasma glucose levels compared to mammals is yet to be determined.

When comparing oxidative stress markers and antioxidant levels in bird compared to rats striking differences are revealed. Studies conducted on the domestic pigeon (*Columba livia*) show resistance to the formation of ROS in brain, heart and kidney tissues despite the naturally high circulating plasma glucose levels of birds compared to rats (Ku & Sohal, 1993). ROS assessed with a fluorescent indicator 7'-dichlorodihydorfluorescein diacetate, acetyl ester in isolated mesenteric arteries from mourning doves (*Zenaida macroura*) compared to rats showed no significant differences (C. L. Smith et al., 2011). Whereas circulating levels of the antioxidant scavengers SOD and catalase were lower in doves compared to rats, the same study found significantly higher levels of plasma uric acid, α-tocoperol (vitamin E), and retinol (vitamin A) (C. L. Smith et al., 2011). These antioxidants may be essential to the protection of avain tissues from oxidative stress in the presence of naturally high blood glucose levels.

EDRF and EDHF and Acute Hyperglycemic Conditions

An experiment conducted on rat epineural arterioles investigated the effect of hyperglycemia on EDRF though NO-mediated vasodilation and EDHF mediated vasodilation in type 1 and type 2 model rats (Coppey, Gellett, & Yorek,

2003). After 4 weeks of hyperglycemia, rats having blood glucose of 300 mg/dl or greater, showed impairment in the EDHF mediated vasodilation, while the NO-mediated vasodilation remained similar to normal control rats. This shows that impaired EDHF signaling or sensitivity, but not impaired NO signaling, was responsible for the endothelial dysfunction resulting from hyperglycemia. The authors of this experiment highlight the potential role of a p450 metabolite, hydrogen peroxide, potassium ion or electrical coupling for the impairment in EDHF signaling (Coppey et al., 2003). Interestingly, an experiment on mice demonstrated impaired endothelium-mediated vasodilation following short-term ingestion of high glucose for both the small mesenteric arterioles and the aorta (Ellis et al., 2008). This experiment showed that both NO and EDHF were impaired among the mice that were hyperglycemic, by using inhibitors of NO and potassium channels during acetylcholine-mediated vasodilation (Ozkan & Uma, 2005). Moreover, an experiment investigating isolated rat mesenteric arteries showed impaired vasodilation through EDHF caused by ROS-mediated effects of hyperglycemia (Ozkan & Uma, 2005). Isolated mesenteric arteries were incubated with a 22.2mM glucose solution for 6 hours. Following the incubation, ACh-mediated vasodilation was assessed in the presence of a NO inhibitor or EDHF inhibitor with or without the antioxidants, superoxide dismutase and catalase. Both NO-mediated and EDHF vasodilation were impaired in response to the hyperglycemic condition, but the impairment was attenuated in the presence of antioxidants. This experiment demonstrated that superoxide ($O_2^{\cdot -}$) and hydrogen

peroxide (H_2O_2) are involved in the impaired vasodilation associated with hyperglycemia.

The vasodilatory responses to EDHF occur through various calcium activated potassium channels: small, intermediate and large conductance channels. It is thought that the large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) play the predominant role in this pathway and potentially hyperglycemia induced oxidative stress alters the normal vascular hyperpolarization. This hypothesis has come from previous investigations showing small and intermediate Ca^{2+}-activated K^+ channel inhibitors did not have an effect on the vasodilatory response to ACh (Mori, Suzuki, Sakamoto, Nakahara, & Ishii, 2011a). The BK_{Ca} inhibitor iberiotoxin was used in diabetic rat retinal arterioles to further examine this hypothesis (Mori, Suzuki, Sakamoto, Nakahara, & Ishii, 2011b). This study induced diabetes with an injection of streptozotocin to male rats and after 2 weeks of persistent hyperglycemia, ACh-mediated vasodilation was assessed compared to age-matched control rats. Since inhibition of BK_{Ca} channels in early stages of diabetes in rats occurred this demonstrates that impairment in this pathway may play an important role hyperglycemia-induced vascular dysfunction (Mori, Suzuki, Sakamoto, Nakahara, & Ishii, 2011b).

Chapter 3

ACETYLCHOLINE-MEDIATED VASODILATION IN SYSTEMIC ARTERIES FROM MOURNING DOVES (Z. MACROURA)

Summary

For mammals, acetylcholine (ACh) promotes endothelium-dependent vasodilation primarily through nitric oxide (NO) and cyclooxygenase (COX)-mediated pathways, with varying reliance on endothelial-derived hyperpolarizing factors. Little to no studies have been conducted on small resistance systemic arteries from birds, which naturally have high blood glucose and fatty acid concentrations that would typically impair endothelium-dependent vasodilation in mammals. Therefore, the hypothesis was that ACh-induced vasodilation of isolated arteries from mourning doves (*Z. macroura*) would be independent of endothelial-derived factors. Small resistance mesenteric and cranial tibial (c. tibial) arteries (80-150 μm, inner diameter) were pre-constricted to 50% of resting inner diameter with phenylephrine then exposed to increasing doses of ACh (10^{-9} to 10^{-5} μM) or the NO donor, sodium nitroprusside (SNP; 10^{-12} to 10^{-3} μM). For mesenteric arteries, ACh-mediated vasodilation was significantly blunted with the potassium channel antagonist tetraethylammonium chloride (TEA, 10 mM); whereas responses were only mildy impaired with endothelial disruption or inhibition of COX (indomethacin; 10 μM). In contrast, endothelial disruption as well as exposure to TEA largely abolished vasodilatory responses to ACh in c. tibial arteries while no effect of COX-inhibition was observed. For both vascular beds, responses to ACh were independent of NO as inhibition of NO synthase had

no impact, despite complete reversal of phenylephrine-mediated tone with SNP. Endothelium-independent vasodilation also relied on potassium channels. In summary, ACh-mediated vasodilation of mesenteric arteries occurs through COX as well as the activation of potassium channels to induce hyperpolarization whereas c. tibial arteries rely primarily on the activation of potassium channels.

Introduction

Endothelial dysfunction in an early predictor of cardiovascular disease that can occur with hyperlipidemia, hyperglycemia and insulin resistance (Baron, 2002; Baron, 2002; Frick & Weidinger, 2007; Kasprzak et al., 2006; Lind, 2006; Potenza et al., 2009) . Birds are of interest in relation to the study of endothelium-dependent vasodilation since they present natural models of resistance to insulin-mediated glucose uptake along with high physiological levels of circulating fatty acids and glucose (Braun & Sweazea, 2008; K. L. Sweazea, Wolf, Braun, & Walker, 2008). In fact, plasma glucose levels for birds are 1.5-2 times levels measured in mammals of similar body mass (Braun & Sweazea, 2008; K. L. Sweazea et al., 2008).

Acetylcholine (ACh) is frequently used to evaluate endothelial function (Drexler & Hornig, 1999; Drexler & Hornig, 1999; Vanhoutte, 1986). Binding of ACh to endothelial muscarinic receptors in arteries activates both nitric oxide (NO)-dependent and independent vasodilatory pathways. Either or both pathways may be impaired with endothelial dysfunction. Through the NO-dependent pathway, ACh triggers an increase in intracellular calcium within endothelial

cells, which activates endothelial nitric oxide synthase (eNOS) to produce the potent endogenous vasodilator NO. The main mechanism by which NO elicits vasodilation is through increased synthesis of cGMP in vascular smooth muscle (VSM) cells leading to decreased cytosolic Ca^{2+} concentrations (Jin & Loscalzo, 2010).

NO-independent pathways of ACh-mediated vasodilation include cyclooxygenase (COX)-mediated production of prostaglandins, which activate adenylate cyclase and cAMP in VSM cells to decrease intracellular Ca^{2+} levels (Jerca et al., 2002). Other NO-independent pathways include those that stimulate hyperpolarization through the activation of a variety of potassium channel subtypes within endothelial and VSM cells. These effects are mediated by endothelial-derived hyperpolarizing factors (EDHFs) (Busse et al., 2002; Edwards et al., 2010; Jackson, 2005). Recent research also shows that endothelial-derived relaxing factors (EDRFs) like NO can activate EDHFs to further promote vasodilation (Jackson, 2005).

For humans, ACh-mediated vasodilation is dependent on NO and the COX derivatives, prostaglandins (Kellogg, Zhao, Coey, & Green, 2005; Medow et al., 2008). In murine skeletal muscle arterioles, NO is the main mediator of ACh-induced vasodilation (Huang et al., 2000). Therefore, impaired NO signaling or reduced bioavailability of this important vasodilator is thought to be one of the main culprits of endothelial dysfunction (Jin & Loscalzo, 2010). In contrast to mammals, studies examining vasodilatory pathways in birds have been limited to domestic species (chickens) as opposed to free living animals as well as the

examination of large conduit blood vessels whereas small resistance arteries play a more direct role in the regulation of blood pressure. Early studies showed that perfused chicken anterior mesenteric and hind-limb (branching from the sciatic a.) arteries respond to ACh with reduced perfusion pressures indicative of vasodilation (A. Knight & McGregor, 1974). For example, studies in chickens have shown that ACh-mediated vasodilation of aortic rings(Hasegawa & Nishimura, 1991)(Hasegawa & Nishimura, 1991), carotid artery (Leo et al., 2008), and pulmonary arteries (Martinez-Lemus, Hester, Becker, Jeffrey, & Odom, 1999; Wang, Wang, Xiang, & Sun, 2002)are dependent on an EDRF. Further studies demonstrated that the response in chicken ductus arteriosus is endothelium-dependent and relies on NO as well as an EDHF (Agren et al., 2008). Therefore, the hypothesis of this study is that because birds have naturally high blood glucose and fatty acid concentrations, ACh-mediated vasodilation in small resistance arteries from doves is expected to be independent of NO and other endothelial-derived factors.

Materials and Methods

Animal Model

Adult male mourning doves (*Z. macroura*; 110-130 g body mass) were collected in Tempe, Arizona using walk-in funnel traps baited with wild birdseed and sunflower seeds. Doves were chosen because of the large population of these birds near the Arizona State University Tempe campus and they have been used in similar studies by our laboratory (C. L. Smith et al., 2011; K. L. Sweazea,

McMurtry, & Braun, 2006). Birds were collected from the same location at approximately the same time of the morning, and transported to the laboratory in cloth bags with drawstring closures to minimize stress. All animal protocols were pre-approved by the Arizona State University Institutional Animal Care and Use Committees and were conducted with necessary permits from relevant agencies.

Analysis of Plasma Nitrates and Nitrites

Blood was collected from a subset of 5 birds by venipuncture for the analysis of plasma nitrates and nitrites (NOx) using a commercially available kit (Cayman Chemical Company, Ann Arbor, MI, USA).

Isolation of Blood Vessels

All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise specified. Animals were euthanized with sodium pentobarbital (200 mg/kg body mass, i.p.), which was ensured by exsanguination secondary to removal of the mesenteric arcade. The mesenteric arcade was removed following a midline laparotomy and pinned out in a silastic-coated dissection dish filled with ice-cold HEPES buffered saline (in mM: 134.4 NaCl, 6 KCl, 1 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.4 with NaOH). The legs were also removed and placed in ice-cold HEPES buffered saline for the extraction of c. tibial arteries. Small resistance mesenteric and c. tibial arteries (~1 mm length; 80-150 µm inner diameter) were isolated and transferred to a HEPES-filled arteriograph (Living Systems Instrumentation, CH-1, Burlington, VT, USA), cannulated with glass

pipettes, and secured in place with silk ligatures (Fig. 1). The vessels were stretched longitudinally to approximate *in situ* length and pressurized to physiologic conditions of 60 mmHg with a servo-controlled peristaltic pump (Living Systems Instrumentation, Burlington, VT, USA). The arteries were then superfused with avian physiological salt solution (APSS; in mM: 114 NaCl, 25 $NaHCO_3$, 10 $NaC_2H_3O_2$, 5 KCl, 2.5 $CaCl_2$, 1 NaH_2PO_4, 0.5 $MgCl_2$, 11.1 glucose) that was aerated to maintain pH and heated to 41˚C (normal dove body temperature).

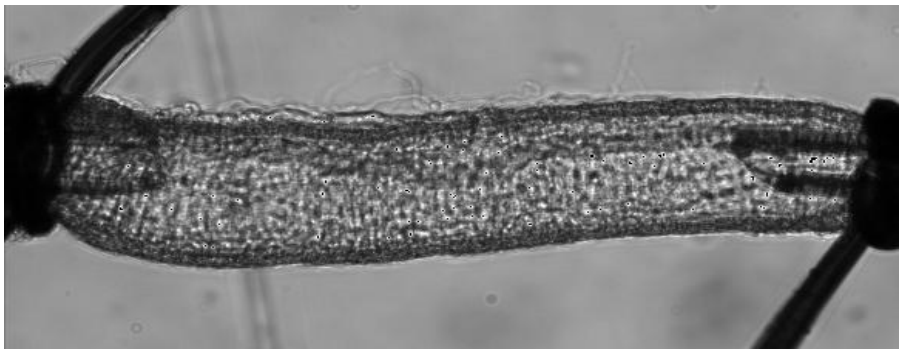

Figure 1. Cannulated and pressurized small resistance mesenteric artery from a mourning dove. Image taken at 10x.

Evaluation of Vasodilatory Pathways

In preliminary studies, the effectiveness of ACh at reversing phenylephrine (PE) and potassium chloride (KCl)-induced vasoconstriction was assessed. Phenylephrine acts through α1-adrenergic receptors to elevate intracellular calcium concentrations, resulting in vasoconstriction (Wier & Morgan, 2003). In contrast, KCl causes vasoconstriction through depolarization of the vascular smooth muscle cells and has been used in prior studies of chicken ductus arteriosus arteries (Agren et al., 2007; Agren et al., 2008). For dove

arteries, ACh fully reversed PE-mediated vasoconstriction (Fig. 2, top panel) but only partially reversed KCl-mediated vasoconstriction (Fig. 2, bottom panel). Therefore, we utilized PE-mediated tone to study the mechanisms of ACh-induced vasodilation.

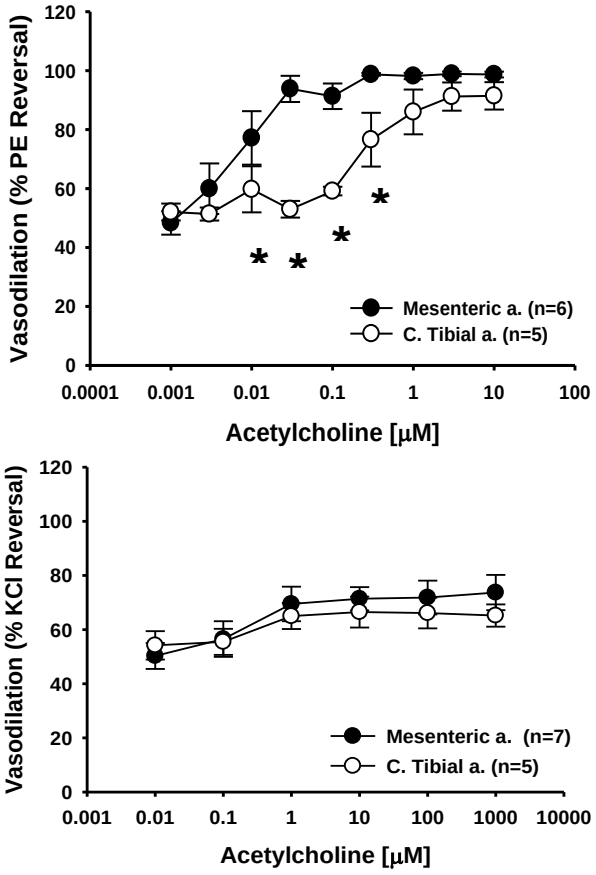

Figure 2. Acetylcholine-mediated vasodilation in isolated small resistance mesenteric and c. tibial arteries from doves. The responses to acetylcholine were measured in vessels pre-constricted to 50% of resting inner diameter with stepwise increases in phenylephrine (top panel) or KCl (bottom panel) in the superfusate. Mesenteric arteries are significantly more sensitive than c. tibial arteries to ACh-mediated reversal of PE-induced tone whereas ACh did not fully reverse KCl-mediated vasoconstriction in arteries from either vessel bed. Data are expressed as means ± s.e.m. *$p < 0.02$ from mesenteric arteries.

After equilibration for 30 minutes in APSS, isolated arteries were superfused for 1 hour with either a control APSS solution or APSS with the addition of antagonists of specific pathways thought to be involved in ACh-mediated vasodilation. Vessels were then pre-constricted to 50% of their resting inner diameter with increasing concentrations of PE (10^{-8} to 10^{-5} M, 5 min each step) in the superfusate followed by exposure to stepwise increases of either ACh (10^{-8} to 10^{-5} M, 5 min each step) or the NO donor sodium nitroprusside (SNP; 10^{-7} to 1M, 5 min each step). To assess reliance on the endothelium, the proximal end of a separate set of arteries was cannulated and the endothelium disrupted by rubbing the lumen with a strand of moose mane and flushing the artery with buffer prior to cannulation of the distal end. The inner diameter was continuously monitored from bright field images using video microscopy and edge-detection software (IonOptix, Milton, MA, USA). Following the concentration response curves, vessels were superfused for 30 minutes with calcium-free APSS (in mM: 114 NaCl, 25 $NaHCO_3$, 10 $NaC_2H_3O_2$, 5 KCl, 1 NaH_2PO_4, 0.5 $MgCl_2$, 11.1 glucose and 3 EGTA) to obtain the passive diameter from which percent reversal of PE-induced tone was calculated.

To characterize ACh-mediated vasodilatory pathways, various antagonists were present in the cannulae throughout the experiment as well as in the superfusate beginning one hour prior to pre-constriction of the vessels with PE. To assess the role of NO in ACh-mediated vasodilation, vessels were pre-exposed to the NO synthase inhibitor Nω-nitro-L-arginine (LNNA; 100 μM) as described in prior studies (K. L. Sweazea et al., 2010). Tetraethylammonium chloride (TEA;

10 mM), a non-specific antagonist of calcium-activated potassium channels (Benham, Bolton, Lang, & Takewaki, 1985; Langton, Nelson, Huang, & Standen, 1991), was used to assess the role of the EDHF pathway (Cook et al., 1992; Jackson, 2005; Pagan et al., 2010). The role of COX in the EDRF pathway of ACh-mediated vasodilation was assessed using the inhibitor indomethacin (10 μM) as described in prior studies (K. L. Sweazea et al., 2010).

Statistical Analysis

For all concentration response curves, data are expressed as the mean ± s.e.m. Data was arcsin transformed to approximate normal distribution prior to statistical analyses and the arcsin-transformed data was analyzed by two-way repeated measures ANOVA. Where significant differences occurred, data was analyzed by Student Newman Keuls posthoc analyses using SigmaStat 3.5 software (Systat Software Inc., Chicago, IL, USA). Significant differences were determined at $p \leq 0.05$.

Results

Plasma nitrate and nitrite (NOx) levels in mourning doves were 15.70 ± 7.22 μM/L, (n=5). Mesenteric arteries were more sensitive to ACh than c. tibial (Fig. 2). Exposure of arteries from both vessel beds to LNNA, a potent NOS inhibitor, did not impact vasodilation (Fig. 3). These findings indicate that ACh acts through NO-independent pathways to cause vasodilation in both mesenteric and c. tibial arteries. In response to the COX inhibitor, indomethacin, ACh-

vasodilation was slightly impaired in mesenteric arteries whereas there were no effects observed in c. tibial arteries (Fig. 4). Therefore, ACh-vasodilation of mesenteric arteries is partially dependent on COX or its product, prostaglandin. ACh-mediated vasodilation was significantly impaired in the presence of the calcium-activated potassium channel antagonist, TEA, in both vessel beds with mesenteric arteries showing greater reliance on these channels compared to c. tibial arteries (Fig. 5).

The role of the endothelium was explored in a separate set of endothelial-disrupted vessels. ACh-mediated vasodilation was moderately impaired in mesenteric arteries with complete vasodilation achieved with higher doses of the vasodilator (Fig. 6). These findings show that vasodilation in mesenteric arteries from doves may not be entirely endothelium-dependent. However, it is possible that residual endothelial cells may remain following disruption of the endothelium resulting in partial activation of endothelium-dependent pathways. In contrast, vasodilation was blocked in endothelial-disrupted c. tibial arteries even at high doses of ACh (Fig. 6). Therefore, c. tibial arteries rely upon the endothelium for ACh-mediated vasodilation.

Smooth muscle sensitivity to NO was assessed in a separate set of mesenteric and c. tibial arteries through dose response curves with the nitric oxide donor, SNP. No significant differences in the responsiveness to SNP were measured between mesenteric and c. tibial arteries (Fig. 7). The role of potassium channels in NO-mediated vasodilation was measured in vessels pre-exposed to TEA. Inhibition of potassium channels significantly impaired vsaodilation in both

mesenteric and c. tibial arteries (Fig. 7) indicating that NO may act directly through potassium channels to elicit vasodilation in small resistance arteries from doves.

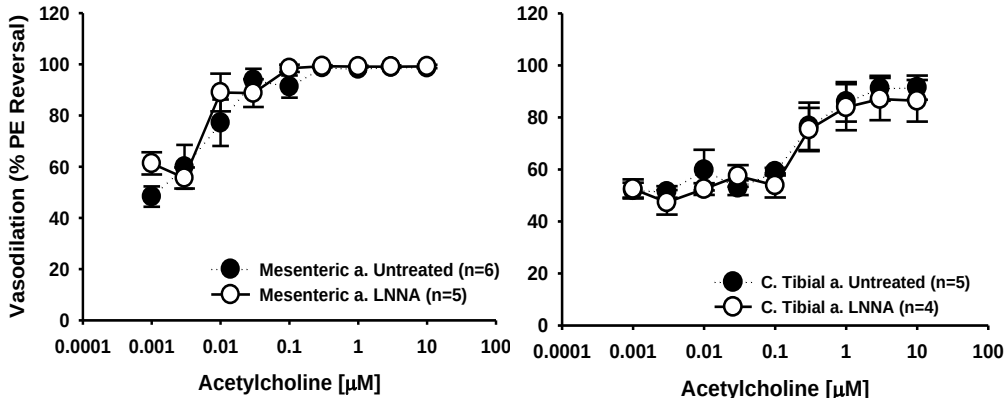

Figure 3. Acetylcholine-mediated vasodilation in isolated small resistance mesenteric and c. tibial arteries following inhibition of nitric oxide synthase with Nω-nitro-L-arginine (LNNA; 100 µM). The cannulae were pre-loaded with ARS containing LNNA during cannulation and LNNA was present in the superfusate one hour prior to and during the concentration response curves. Data from untreated vessels (dotted lines) are repeated from figure 2 for comparison. There are no significant differences between acetylcholine-induced vasodilation for untreated and LNNA-treated arteries. Data are expressed as means ± s.e.m.

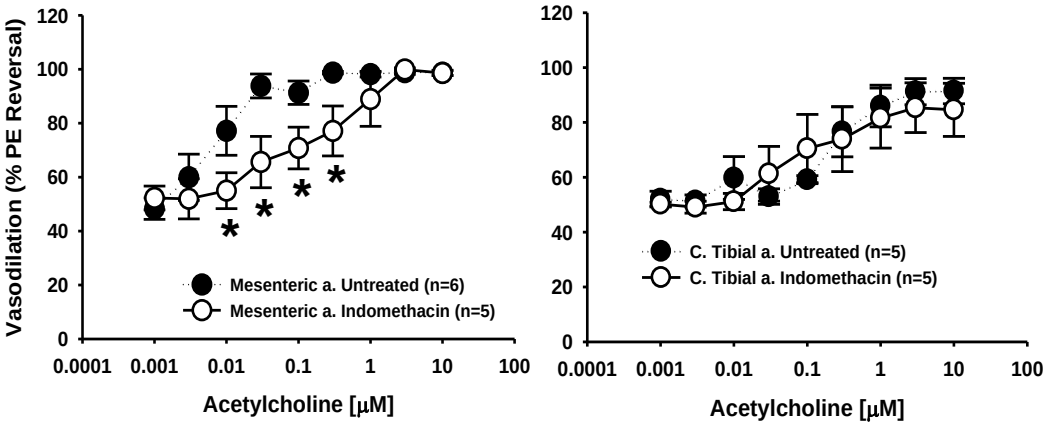

Figure 4. Acetylcholine-mediated vasodilation in isolated small resistance mesenteric and c. tibial arteries following inhibition of cyclooxygenase with indomethacin (10 μM). The cannulae were pre-loaded with ARS solution containing indomethacin during cannulation and the superfusate was loaded with indomethacin one hour prior to and during the concentration response curves. Data from untreated vessels (dotted lines) are repeated from figure 2 for comparison. Data are expressed as means ± s.e.m. *$p < 0.02$ from respective untreated controls

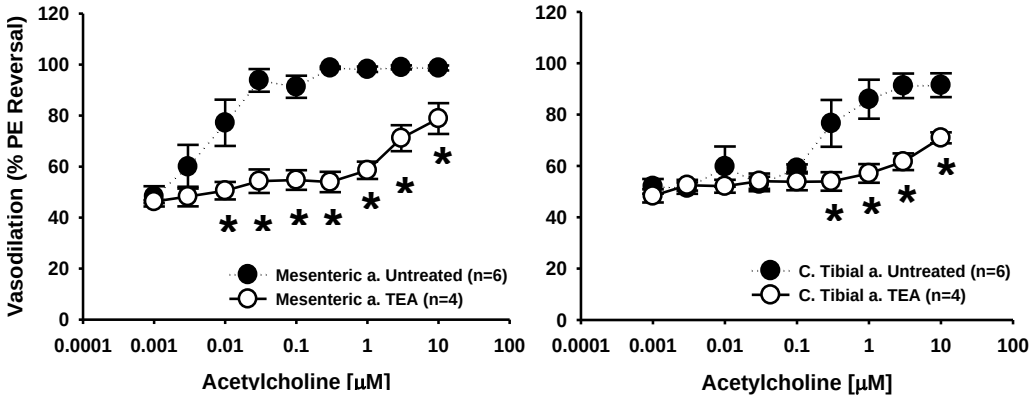

Figure 5. Acetylcholine-mediated vasodilation in small resistance isolated mesenteric and c. tibial arterioles from doves following incubation with an inhibitor of calcium-activated potassium channels, tetraethylammonium chloride (TEA; 10 mM). Arteries were pre-treated with TEA in the lumen (during cannulation) and superfusate for one hour prior to and during the concentration response curves. Data from untreated vessels (dotted lines) are repeated from figure 2 for comparison. Data expressed as means ± s.e.m. *$p \leq 0.001$ from respective untreated controls.

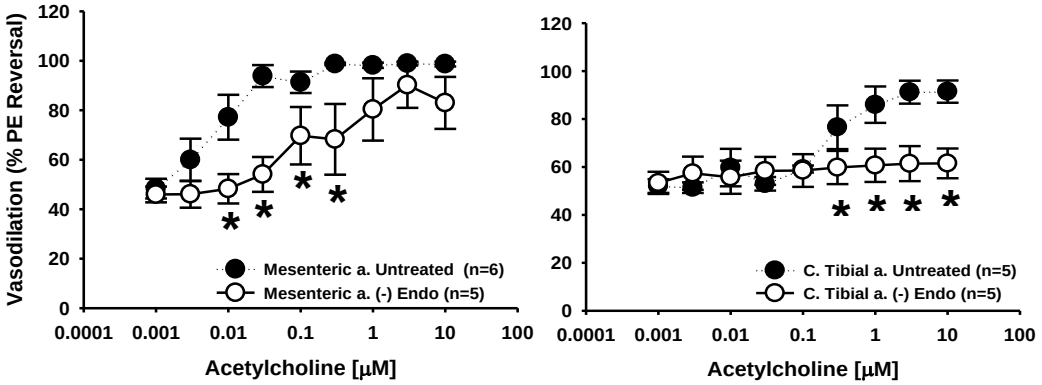

Figure 6. Role of the endothelium in acetylcholine-mediated vasodilation of small resistance mesenteric and c. tibial arteries from doves. The endothelium was disrupted by rubbing the lumen with moose mane during cannulation as described in the methods. Data from untreated vessels (dotted lines) are repeated from figure 2 for comparison. Date expressed as means ± s.e.m. *$p < 0.05$ from respective untreated controls.

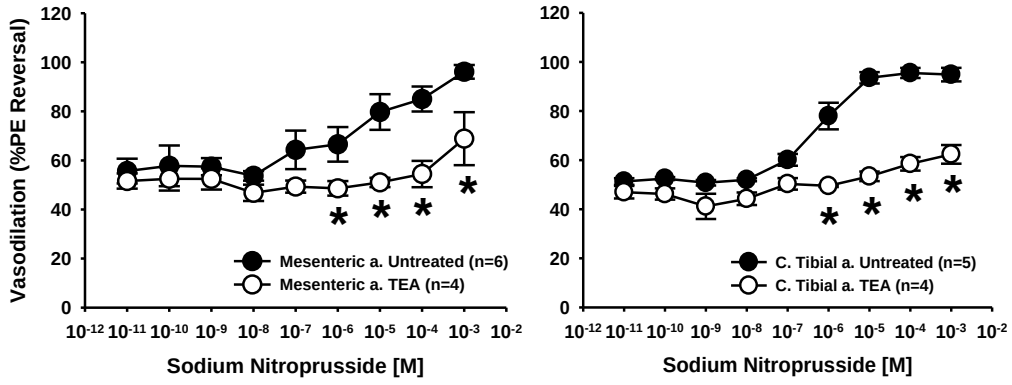

Figure 7. Smooth muscle sensitivity to the NO donor sodium nitroprusside in isolated small resistance mesenteric and c. tibial arterioles from doves. The responses to sodium nitroprusside were measured in vessels pre-constricted to 50% of resting inner diameter with stepwise increases in phenylephrine in the superfusate. There are no significant differences in vascular smooth muscle sensitivity to NO between mesenteric and c. tibial arterioles. Data expressed as means ± s.e.m. *$p \leq 0.05$ from respective untreated controls.

Discussion

The major results from this study show that 1) mesenteric are more sensitive to ACh than c. tibial arteries; 2) ACh-induced vasodilation of both mesenteric and c. tibial arteries is mediated by potassium channels, not NO, although NO donors are effective at directly eliciting vasodilation through the activation of potassium channels; 3) vasodilation in mesenteric arteries is also partially-dependent on the EDRF, cyclooxygenase; and 4) vasodilation in c. tibial arteries is endothelium-dependent.

Studies of large arteries show variations in the dependence of ACh-mediated vasodilation on NO-activated pathways across vertebrates. For example,

ACh-mediated vasodilation of the cerebral circulation of crucian carp (*C. carassius*; (Hylland & Nilsson, 1995)) and aorta of leopard frogs (*R. pipiens*, (G. E. Knight & Burnstock, 1996)) rely on NO-dependent pathways. Similarly, NO directly dilates pre-constricted dorsal aorta from short-finned eels (*A. australis*) and the coronary system of trout (*O. mykiss;* (Donald & Broughton, 2005; Mustafa, Agnisola, & Hansen, 1997)). For short-finned eels and cane toads (*B. marinus*), although ACh-mediated vasodilation is NO-dependent, it does not require an intact endothelium. Rather, it has been shown to activate neuronal NOS (nNOS) to produce NO as opposed to eNOS (Donald & Broughton, 2005). In contrast to these animals, no evidence for a NO-dependent signaling mechanism was found in giant shovelnose rays (*R. typus*; (Donald & Broughton, 2005)) or dogfish sharks (*S. acanthias;* (Evans & Gunderson, 1998)). Moreover, ACh produces a concentration-dependent contraction of the ventral aorta from dogfish sharks that is not endothelium-dependent (Evans & Gunderson, 1998). This is similar to what has been observed in freshwater eels (*A. 38nguilla*), and trout (*S. gairdneri*) in which ACh or NO-donors caused vasoconstriction (Donald & Broughton, 2005; Pellegrino, Sprovieri, Mazza, Randall, & Tota, 2002).

For doves, ACh-mediated vasodilation is not dependent on NO as incubation of arteries with the non-specific inhibitor of NOS, LNNA, had no effect on vasodilation (Fig. 3). This is in contrast to chickens for which ACh-mediated vasodilation appears to be NO-dependent. Pulmonary hypertension is commonly diagnosed in broiler chickens whereas Leghorn chickens are resistant to this disorder. Research has shown that ACh-mediated vasodilatory responses

are lower in pre-constricted pulmonary artery rings from broiler compared to Leghorn chickens as a result of lower levels of NO activity (Martinez-Lemus et al., 1999). Moreover, intravenous infusion of a NOS inhibitor in broiler chickens decreases plasma NO concentrations and increases pulmonary arterial pressure (Wang et al., 2002) further supporting a diminished role for NO in these birds, although the pulmonary circulation can be quite different from systemic arteries. In other studies, inhibition of NOS reduces the sensitivity of chicken carotid artery segments to ACh but not the efficacy of ACh at dilating arteries. Further research showed that the L-NAME resistant portion of the vasorelaxation was attributed to carbon monoxide (Leo et al., 2008), which is another EDRF that has recently been shown to activate large conductance calcium-activated potassium channels (Bolognesi et al., 2007).

Isolated White Leghorn chicken ductus arteriosus artery rings exhibit dual responses to ACh in which low concentrations (30nM-1 µM) elicit vasodilation and high concentrations (3µM-0.1 mM) have the opposite effect. However, both effects are endothelium-dependent and ACh-mediated vasocontraction occurred through COX (Schuurman & Villamor, 2010). A similar vasoconstrictor role for ACh at higher doses was not seen in the small resistance arteries from doves examined in the present study (Fig. 2) although ACh-vasodilation relied partially on COX in mesenteric arteries (Fig. 4). This is similar to observations of ACh-vasodilation of aortas isolated from dogfish sharks and shovelnose rays, which rely on COX-dependent mechanisms (Donald, Broughton, & Bennett, 2004; Evans & Gunderson, 1998). The lower sensitivity of c. tibial arteries to ACh was

not attributed to ACh-mediated vasoconstriction through COX since inhibition with indomethacin did not improve the response (Fig. 4).

In the current studies, ACh was less effective at reversing KCl compared to phenylephrine-induced vasoconstriction supporting a role for potassium channels in the response (Fig. 2, bottom panel). Similar findings were observed in chicken ductus arteriosus rings that were pre-contracted with KCl versus phenylephrine and stimulated to relax using hydrogen sulfide (van der Sterren, Kleikers, Zimmermann, & Villamor, 2011). When vessels from doves were pre-incubated with the calcium-activated potassium channel inhibitor TEA, ACh- as well as NO-mediated vasodilation responses were significantly blunted in both vascular beds (Figs 5, 7). Therefore, both ACh and NO elicit vasodilation through the activation of potassium channels in mesenteric and c. tibial arteries of doves. Studies of isolated rat mesenteric arteries show that ACh activates factors that stimulate potassium channels within the endothelium and VSM cells resulting in hyperpolarization and vasodilation. Activation of hyperpolarization of VSM cells can be induced directly (as in the case of endothelium-denuded arteries) or indirectly through myoendothelial gap junctions (Dora, Gallagher, McNeish, & Garland, 2008). Since disruption of the endothelium abolished vasodilation in c. tibial arteries, ACh and NO likely act directly on VSM cell potassium channels. Direct activation of VSM cell potassium channels are also implicated in the vasodilatory responses of mesenteric arteries since disruption of the endothelium only partially impaired vasodilation (Figs 5, 6). However, it is possible that residual endothelial cells remained a source of hyperpolarizing or relaxing factors.

These findings are similar to studies of skeletal muscle arterioles from eNOS knockout mice in which activation of potassium channels becomes the predominant vasodilatory mechanism in contrast to wild type mice in which NO is the main contributor (Huang et al., 2000).

In conclusion, ACh-mediated vasodilation of mesenteric arteries from doves is dependent on potassium channels and COX, whereas c. tibial arteries rely mainly on potassium channels. For both vascular beds, ACh and NO may act directly to hyperpolarize vascular smooth muscle cells through the activation of potassium channels. The NO-independent nature of ACh-mediated vasodilation in arteries from doves with naturally high blood glucose levels is similar to what is seen in mammals that are hyperglycemic and likewise resistant to the glucose lowering effects of insulin (Baron, 2002; Braun & Sweazea, 2008).

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Chapter 4

ACUTE HYPERGLYCEMIC CONDITIONS IMPAIR ACETYLCHOLINE-MEDIATED VASODILATION IN ISOLATED ARTERIES FROM MOURNING DOVES (Z. MACROURA)

Summary

Normal avian plasma glucose levels are 1.5-2 times greater than mammals of similar size. In mammals, hyperglycemia induces oxidative stress and leads to impaired endothelium-dependent vasodilation. Prior work has shown that birds have high levels of antioxidants. Therefore, the hypothesis was that endothelium-dependent vasodilation of isolated avian arteries would not be impaired following acute exposure to high glucose. Isolated small resistance cranial tibial arteries (c. tibial; 80-150μm, inner diameter) were cannulated and pressurized in a vessel chamber then incubated with a physiological saline solution containing either normal or high glucose (20mM vs. 30mM) for 1 hour at 41°C. Vessels were then pre-constricted to 50% of resting inner diameter with phenylephrine (PE) followed by increasing doses of acetylcholine (ACh; 10^{-9} to 10^{-5} M, 5 min per step). Percent reversal of PE-induced tone was measured by tracking the inner diameter with edge-detection software. Contrary to our hypothesis, ACh-induced vasodilation was impaired with acute exposure to high glucose ($p=0.013$). The impairment was not related to increased osmolarity since vasodilation of arteries exposed to an equimolar combination of 20mM glucose and 10mM mannitol was not different from controls ($p=0.898$). Rather, the impaired vasodilation was attributed to oxidative stress since superoxide levels were elevated 168 ± 42%

(P=0.0072) in arteries exposed to high glucose and pre-exposure of arteries to the superoxide dismutase mimetic tiron (10mM) improved vasodilation ($p<0.05$). Therefore, isolated arteries from doves do not appear to have endogenous mechanisms to prevent impaired vasodilation resulting from high glucose mediated increases in oxidative stress.

Introduction

Endothelial dysfunction of the microvascular system is a major complication of diabetes and a predictor of future cardiovascular disease (de Haan & Cooper, 2011; Sitia et al., 2010). The deleterious effect of hyperglycemia on normal vascular function is well-documented in mammals. Preceding clinical symptoms of vascular disorders, hyperglycemia impairs endothelium-mediated vasodilation by altering nitric oxide (NO), prostacyclin (PGI_2), and endothelial derived hyperpolarizing factor (EDHF) dependent pathways (Tsai, Hein, Kuo, & Yang, 2011). Chronic and acute hyperglycemia increase the production of reactive oxygen species (ROS), resulting in the development of oxidative stress, which is a well-known cause of endothelial dysfunction (de Haan & Cooper, 2011).

The source of ROS in the vasculature consists of enzymatic and non-enzymatic sources such as NADPH oxidase, the mitochondrial electron transport chain, xanthine oxidase and nitric oxide synthase (Fatehi-Hassanabad, Chan, & Furman, 2010). In a hyperglycemic state, superoxide ($O_2^{\cdot-}$) production from mitochondrial respiration becomes elevated. ROS-induced vascular dysfunction

occurs through impairment of endothelial-derived relaxing factors such as NO, and endothelial-derived hyperpolarizing factors (EDHF) (Fatehi-Hassanabad et al., 2010; Potenza et al., 2009). Because $O_2^{\cdot -}$ is a potent scavenger of NO, overproduction of this ROS results in reduced bioavailability of NO and impaired vasodilatory reactivity. Hyperglycemia also promotes increased production of intracellular advanced glycation end products (AGE), thereby promoting a proinflammatory state (Inoguchi et al., 2003; Kojda & Harrison, 1999; Yao & Brownlee, 2010). EDHF-mediated dilation has been described as a compensatory mechanism to induce vasodilation when NO activity is impaired (Ozkan & Uma, 2005). However, EDHF-mediated relaxation is also diminished in the presence of high glucose. This may be though reduced cytochrome p450 epoxygenase activity, which subsequently reduces endothelial epoxyeicosatrienoic acid production (Tsai et al., 2011).

Acetylcholine (ACh) is commonly used to assess endothelium-vasodilation (Schuurman & Villamor, 2010; Taylor, Graves, & Poston, 1995). ACh activates vasodilation in arteries through stimulation of NO, prostacyclin and EDHF pathways (Kellogg et al., 2005; Medow et al., 2008). Prior studies in our laboratory have shown that ACh-mediated vasodilation is endothelium-dependent in isolated c. tibial arteries from doves, but does rely on either the NO or COX-dependent pathways. Rather, the main contributors to ACh-vasodilation are EDHFs that stimulate calcium-activated potassium channels (Jarrett et al., In Review).

The use of an avian model is a novel approach to study the pathways implicated in acute hyperglycemic condition, because avian plasma glucose concentrations are normally 1.5-2 times greater than that of mammals of similar body mass (Braun & Sweazea, 2008). In fact, levels for mourning doves, specifically, are 18.9 ± 2.9 mM/L (C. L. Smith et al., 2011). Despite this, studies have shown that mitochondria isolated from brain, heart and kidney tissues from pigeons produce significantly less $O_2^{\cdot -}$ and H_2O_2 compared to the same tissues isolated from rats (Ku & Sohal, 1993). Moreover, prior studies from mourning doves have shown that these birds have very high antioxidant capacities and levels of circulating uric acid along with vitamins A and E (C. L. Smith et al., 2011). Therefore, the hypothesis of the current study is that the high endogenous antioxidant capacity of birds protects from hyperglycemia-induced tissue damage thereby maintaining normal vascular function in *ex vivo* arteries exposed to high glucose conditions.

Materials and Methods

Animal Model

Adult male and female mourning doves (*Z. macroura*; 110-130g body mass) were collected in Tempe, Arizona using walk-in funnel traps baited with wild birdseed and sunflower seeds. Doves were chosen because of the large population of these birds near the Arizona State University Tempe campus and they have been used in similar studies by our laboratory (C. L. Smith et al., 2011; K. L. Sweazea et al., 2006). Birds were collected from the same location at

approximately the same time of the morning, and transported to the laboratory in cloth bags with drawstring closures to minimize stress. All animal protocols were approved by the Arizona State University Institutional Animal Care and Use Committee and were conducted with permits from the Arizona Game and Fish Department and US Fish and Wildlife Service.

Isolation of blood vessels

Animals were euthanized with sodium pentobarbital (200 mg/kg body mass, i.p.), which was ensured by exsanguination. The legs were removed and place in ice-cold HEPES buffered saline (in mM; 134.4 NaCl, 6 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, 20 glucose, pH 7.4 with NaOH) for extraction of c. tibial arteries. Small resistance c. tibial arteries (~1mm length; 80-150 µm inner diameter) were isolated and transferred to a HEPES-filled vessel chamber (Living Systems Instrumentation, CH-1, Burlington, VT, USA), cannulated with glass pipettes, and secured in place with silk ligatures on each cannula. The vessels were stretched longitudinally to approximate *in situ* length and pressurized to physiologic conditions of 60 mmHg with a servo-controlled peristaltic pump (Living Systems Instrumentation, Burlington, VT). The arteries were then superfused with avian physiological salt solution (APSS; in mM: 114 NaCl, 25 $NaHCO_3$, 10 $NaC_2H_3O_2$, 5 KCl, 2.5 $CaCl_2$, 1 NaH_2PO_4, 0.5 $MgCl_2$, 20 glucose) that is aerated to maintain pH and heated to 41°C.

Endothelium-dependent Vasodilation

After equilibration for 30 minutes in APSS, isolated c. tibial arteries were superfused for 1 hour with normal APSS or APSS containing 30mM glucose in the presence and absence of the superoxide mimetic 4,5-dihydroxy-1, 3-benzene-disulfonic acid disodium salt (tiron; 10mM). The 20mM glucose solution was chosen as the control, because it is similar to normal physiological plasma glucose concentrations measured for mourning doves (17-10mM/L) (Braun & Sweazea, 2008; C. L. Smith et al., 2011). Vessels were pre-constricted to 50% of their resting inner diameter with increasing concentrations of PE (10^{-8} to 10^{-5} M, 5 min each step) in the superfusate followed by exposure to stepwise increases of ACh (10^{-8} to 10^{-5} M, 5 min each step). Separate arteries were pre-exposed to APSS containing 20mM glucose along with 10mM mannitol to measure the contribution of increased osmolarity to vasoreactivity. The inner diameter was continuously monitored from bright field images using video microscopy and edge-detection software (IonOptix, Milton, MA, USA). Following the concentration response curves, vessels were superfused for 30 minutes with calcium-free APSS (in mM: 114 NaCl, 25 $NaHCO_3$, 10 $NaC_2H_3O_2$, 5 KCl, 1 NaH_2PO_4, 0.5 $MgCl_2$, 11.1 glucose and 3 EGTA) to obtain the passive inner diameter from which percent reversal of PE-induced tone was calculated.

Measurement of Superoxide Production

Vascular superoxide levels from the c. tibial arteries were assessed by using dihydroethidium (DHE) microfluorography. In the presence of superoxide, DHE will oxidize to form the fluorophore ethidium bromide (EtBr), which

intercalates with DNA becoming trapped in the nuclei. A modified protocol from Bagi et al. (Bagi, Koller, & Kaley, 2003) was implemented to test differences in superoxide production from isolated c. tibial arteries exposed to HEPES buffer containing normal or high glucose concentrations. Isolated c. tibial arteries from mourning doves were transferred to microcentrifuge tubes containing HEPES buffer with either 20mM or 30mM glucose and incubated for 60 minutes at 41°C. The vessels were subsequently transferred to a HEPES-filled tube containing DHE (10 µM/ml, Molecular Probes, Invitrogen, Grand Island, NY, USA) and allowed to incubate at 41°C for 10 minutes. Following a 5-minute wash in normal HEPES, the isolated arteries were embedded in OCT and snap-frozen in isopropyl alcohol cooled by dry ice and stored at -80°C until sectioning.

All subsequent steps prior to data analysis were protected from light. Frozen sections were cut at 12µm, collected on Histobond® glass slides (VWR International, Randor, PA, USA), and immediately fixed for 30 minutes in 4% formaldehyde prepared in 100mM phosphate-buffered saline (PBS, pH 7.0). After a 10-minute incubation in PBS, slides were mounted in Vectashield Mounting Medium (Vector Laboratories), and the coverslips sealed with nail polish. Images were collected on either a Leica SP2, or SP5 laser scanning confocal microscope (Leica Microsystems, Buffalo Grove, IL, USA) housed in the W.M. Keck Bioimaging Facility at Arizona State University. Briefly, images were scanned using a 63x, 1.4 NA objective at 0.5 µm increments in the z-axis through the entire volume of the section. To accurately assess pixel intensities, all images were collected at the same magnification under standardized user setting. Pixel

intensity was measured using NIH Image J Software (http://rsbweb.nih.gov/ij/) by averaging three to four different regions of interest, and the percent difference in DHE fluorescence between the arteries pre-exposed to HEPES containing 20mM or 30mM glucose were determined.

Statistical analysis

For all concentration response curves, data are expressed as the mean ± SEM. Data was arcsin transformed to approximate normal distribution prior to statistical analyses and the arcsin-transformed data analyzed by two-way repeated measures ANOVA. Where significant differences occurred, data was analyzed by Student Newman Kuels posthoc analysis using SigmaStat 3.5 software (Systat Software Inc., Chicago, IL, USA). Untransformed data is shown in the graphs. Fluorescence intensity for DHE-stained arteries was quantified using NIH Image J software (http://rsbweb.nih.gov/ij/) and analyzed by two-way ANOVA. Significant differences were determined at $p \leq 0.05$.

Results

The effect of acute exposure to high glucose on vascular function was studied on *ex vivo* c. tibial small resistance arteries. C. tibial arteries were chosen for these studies because skeletal muscle arteries are critical for glucose delivery to myocytes, the main tissue for postprandial glucose disposal (Jackson, 2005). Pre-incubation of arteries for 1 hour in APSS containing 30mM glucose resulted in significantly impaired vasodilation in response to ACh compared to the control

solution containing 20mM glucose ($p<0.05$; Fig. 1). This impaired response was not attributed to an increase in osmolarity since pre-exposure of separate isolated vessels to an equimolar concentration of 20mM glucose plus 10mM mannitol showed no difference in vasodilatory responses from control arteries (data not shown). In contrast, pre-exposure of c. tibial arteries to the superoxide dismutase mimetic tiron normalized the response in arteries exposed to high glucose conditions (Fig. 1). These findings support a role for $O_2^{\cdot -}$ in the impaired vasodilation observed in *ex vivo* arteries from doves exposed to high glucose conditions.

Further support for a role of increased $O_2^{\cdot -}$ comes from vessels pre-exposed to either 20mM or 30mM glucose then loaded with the cell-permeable indicator of $O_2^{\cdot -}$, dihydroethidium. In these studies, superoxide production was shown to be increased by 168.9 ± 42% ($p=0.0072$, $n=5$) in c. tibial arteries after incubation with 30mM glucose compared to paired arteries exposed to 20mM glucose (Fig. 2).

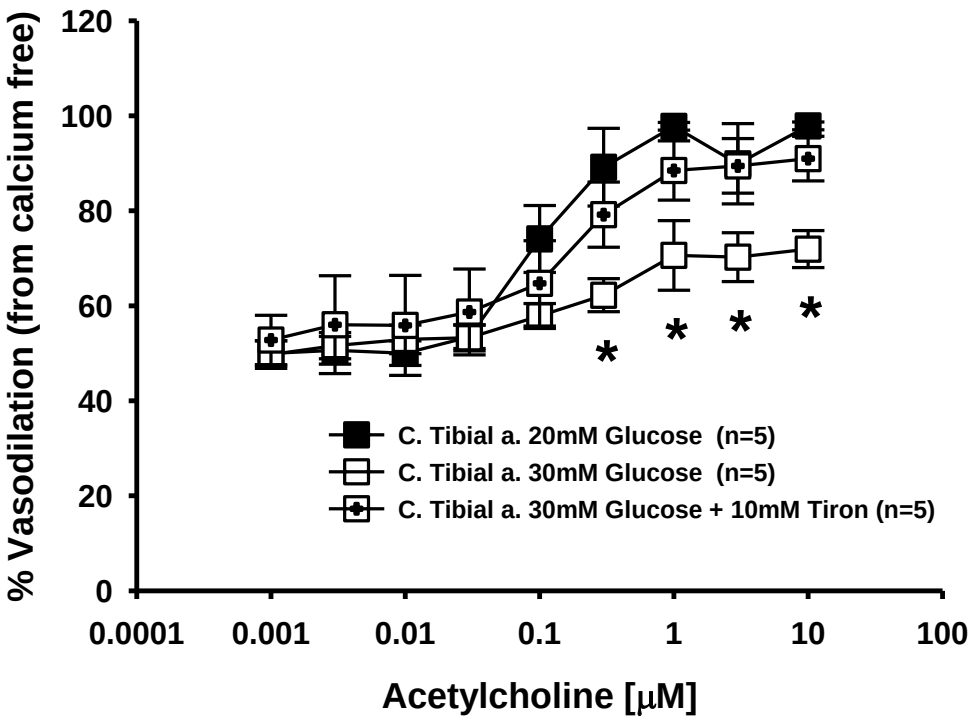

Figure 1.
Acetylcholine-mediated vasodilation in isolated small resistance c. tibial arteries from doves. The responses to acetylcholine were measured in vessels pre-constricted to 50% of resting inner diameter with stepwise increases in phenylephrine in the superfusate. Response curves were conducted in the presence of normal glucose (20mM), high glucose (30mM), and high glucose with the addition of 10mM Tiron. Data expressed as means ± s.e.m. *$p \leq 0.05$ from the respective untreated controls.

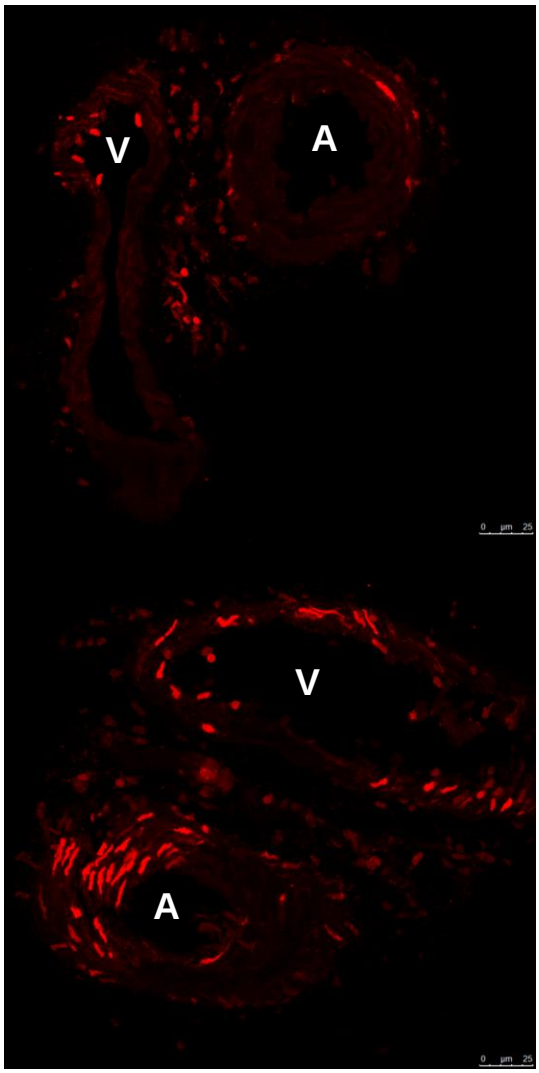

Figure 2.
Representative images of paired vessels from a mourning dove exposed to either control (20mM; top panel) or high (30mM; bottom panel) glucose conditions and stained with dihydroethidium. Ethidium bromide flourescence was significantly higher in arteries exposed to high glucose (168.9 ± 42%, p=0.0072, *n*=5). A=artery, V=vein; scalebar represents 25µm.

Discussion

To our knowledge, this is the first study designed to characterize whether isolated small resistance avian arteries have endogenous mechanisms to protect

vasodilation in the presence of acute hyperglycemic conditions. Contrary to our hypothesis, the main findings of this study are 1) vasodilation of c. tibial arteries from mourning doves is not resistant to acute hyperglycemic exposure (Fig. 1) and 2) exposure of arteries to high glucose conditions results in increased production of $O_2^{\cdot -}$, which is implicated in the impaired vasodilation response following exposure to high glucose (Figs. 1 & 2).

Birds are of particular interest because they are generally long-lived and age slowly compared to mammals. Moreover, studies have demonstrated that birds have more effective resistance against oxidative damage (Holmes & Ottinger, 2003). Doves, pigeons and hens have the same relationship between resting log energy expenditure per day compared to log body weight as mammals (rat, dog, sheep, cow, steer, humans) (Keesey, 1993). However, when examining the basal metabolic rate to maximum lifespan for birds compared to mammals, there is a considerable difference (MLSP birds = 346. $BMR^{-0.51}$; mammals = 270. $BMR^{-0.61}$) with birds having significantly longer lifespans, but this only explains about one quarter of the variation (Hulbert, 2010).

Although doves have higher plasma glucose levels compared to rats (C. L. Smith et al., 2011), the level of vascular ROS are similar (Smith, C.L., Braun, E.J., Sweazea, K.L., 2009). Conversely, mourning doves have significantly higher total plasma antioxidant capacity, uric acid, retinol (vitamin A), and α-tocopherol (vitamin E) (Smith, C.L., Braun, E.J., Sweazea, K.L., 2009). Therefore, we had hypothesized that the endogenous antioxidant capacity of birds could be a mechanism that limits oxidative stress-related damage associated with high blood

glucose. Another possible contributor to protection from oxidative damage, is that avian erythrocytes have a higher turnover rate (~21 days) compared to mammals (~120 days) presumably resulting in lower levels of protein glycation (Hargrove, 2005).

In mammals, exposure to elevated blood glucose results in vascular impairments stemming from endothelial dysfunction mediated by disruption of EDRF and EDHF pathways (Potenza et al., 2009; Tsai et al., 2011). Previous findings from our laboratory have demonstrated that ACh-mediated vasodilation or c. tibial arteries from doves is primarily NO and COX-independent, with vasodilation mainly occurring through EDHF (Jarrett et al., In Review). Consistent with mammals, vasodilation in dove arteries is impaired with acute exposure to high glucose (Fig. 1). Because of the reliance of ACh-vasodilation on EDHFs (Jarrett et al., In Review), it is suspected that this impairment is primarily due to altered EDHF signaling or sensitivity.

In summary, c. tibial arteries from doves do not have intrinsic mechanisms to attenuate impaired vasodilation caused by acute hyperglycemia. Additionally, similar to prior observations in mammas, the impaired vasodilatory response is mediated through increased production of ROS.

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Chapter 5

CONCLUSIONS AND APPLICATIONS

Two important investigations were completed in small resistance arteries of mourning doves. First, the characterization of their dependence on endothelial-derived relaxing factors and endothelial-derived hyperpolarizing factors were determined and secondly, the response to an acute exposure to glucose was examined. There is a general assumption that endothelium-dependent vasodilation is primarily dependent on nitric oxide, with emerging evidence that supports the role of EDHF playing a more predominant role in the microcirculation in mammals. Acetylcholine-mediated vasodilation of mesenteric and c. tibial arteries in mourning doves demonstrates similar observations. Future investigation into the exact mechanisms of the imprecise term EDHF-induced vasodilation needs to be empirically determined in both mammals and birds under normal and stressed states. Mourning dove c. tibial arteries were not resistant to oxidative stress-induced impairment to ACh-mediated vasodilation. The impaired vasodilatory responses were due to increased superoxide production caused by pre-exposure to high glucose conditions.

The overarching investigation of comparative physiology in birds stems from the evidence regarding their known longevity and elevated plasma glucose levels for most avian species compared to mammals. Diabetes is well documented to increase the risk of cardiovascular mortality and morbidity. One of the major hypotheses is hyperglycemia induced oxidative stress leads to endothelial dysfunction, which is the first step in the pathologenesis of atherosclerosis.

Investigating the mechanisms of endothelium-mediated vasodilation in mourning dove arteries may lead to advancements or approaches in the treatment of hyperglycemia-mediated pathologies by building on investigations into the mechanisms that have evolved in birds for protection against oxidative stress.

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APPENDIX A

ISOLATION OF BLOOD VESSELS

Procedure

1. Prepare for experiment:

    a. Turn on recirculation water baths to warm up.

    b. Flush out recirculating tubing with 1L of deionized (DI) water at 20mL/min. This water may go down the drain. Adjust the flow to 10mL/min after water had cleared.

    c. Prepare agonist/antagonist solutions for superfusion of blood vessels as required by each protocol.

    d. Make serial dilutions of vasoactive agents according to each protocol.

    e. Rinse out pressure servo controller tubing by flushing with DI water using a syringe. This water may go down the drain.

    f. Prepare vessel chamber by filing tubing with APSS solution (if loading the vessel lumen with any drugs, add them to the cannula now); make sure to close the stopcocks to prevent air getting into the tubing and disrupting the endothelium unintentionally.

    g. Turn on all computer components.

    h. Fill large rectangular ice bucket with ice; fill two small square ice buckets and make sure the small ice buckets are well-packed with ice and place them in the -20ºC freezer.

    i. Prepare for surgery: Plastic coated pad, surgical instruments, small specimen jar for tissues filled with HEPES buffer, spray

water bottle, 1mL syringe and sodium pentobarbital, 4x4 gauze pads, tubes for extra tissues, dissection dishes filled with HEPES buffer, small piece black plastic bag for placing on top of ice dissection block.

2. Deeply anesthetize bird using 200mg/kg sodium pentobarbital.

3. Isolate appropriate blood vessel euthanizing the animal by exsanguination.

    a. Extract heart, liver, kidney, adipose, pancreas, skeletal muscle tissues as needed for other protocols to minimize animal usage.

    b. Wrap up animal carcass in plastic backed pad and place in labeled bag in -80ºC freezer.

4. Carefully cannulate proximal end of vessel using silk ligatures.

    a. If preparing endothelium-disrupted arteries, insert a single strand of moose main hair into the free distal end of the vessel attached at the proximal end to a cannula. After disrupting the endothelium by rubbing the lumen with the hair, flush the lumen of the artery with APSS.

5. Carefully cannulate distal end of vessel using prepared silk ligatures. Set intraluminal pressure to 60mmHg, and check for leaks in vessel using peristaltic pump on manual.

6. Wash out vessel by superfusing with APSS for 15 minutes into waste bottle. All superfusate solutions are aerated and warmed to 41ºC at a rate of 10mL/min.

7. Record starting inner diameter (ID).

8. Equilibrate for 30 minutes in recirculating APSS.

9. Circulate with drugs in APSS for 1 hour (or plain APSS for controls).

10. Perform desired concentration response curve; record ID after each addition.

11. Wash out with calcium free APSS for 15 minutes into waste bottle.

12. Recirculate with calcium free APSS for 15 minutes; record ID.

13. Rinse all tubing and vessel chamber parts following the curves. Sanitize and rinse all surgical instruments. This waste goes into properly labeled waste containers. Sharps (needles and pipette tips) always go into red plastic sharps containers.

14. Dispose of all gloves, tubes and other waste in appropriate receptacle.

Safety

1. Make sure to wear lab coat and gloves during all procedures. Surgical face masks are to be worn during surgeries to protect against animal dander.

2. Pay careful attention to safety when working with sharp surgical tools.

3. If bitten by an animal the PI is to be contacted immediately, who will then contact Environmental Health and Safety and go to student health for treatment. Since birds do not have teeth, it is not anticipated that this will be an issue.

APPENDIX B

CONCENTRATION RESPONSE CURVES: PHENYLEPHRINE AND ACETYLCHOLINE

Phenylephrine (PE) Concentration Response Curve

Preparation of PE serial dilution: Add 900µL of APSS to five 1ml tubes. Add 100µL of 1M stock PE to the first tube; final concentration 10^{-1} PE. Vortex the tube, and add 100µL of the 10^{-1} PE to the second tube; final concentration 10^{-2}PE. Vortex the second tube, and repeat previous steps until 10^{-5} PE concentration tube is mixed.

Add the following concentrations of PE using the prepared serial dilutions to the same 200mL APSS solution. Record ID after each addition. Add the concentrations of PE until an inner diameter of 50% of resting is achieved.

1. Add 20µL of 10^{-5} PE; Final concentration: 10^{-9} PE
2. Add 40µL of 10^{-5} PE; Final concentration: $3x10^{-9}$ PE
3. Add 140µL of 10^{-5} PE; Final concentration: 10^{-8} PE
4. Add 40µL of 10^{-4} PE; Final concentration: $3x10^{-8}$ PE
5. Add 140µL of 10^{-4} PE; Final concentration: 10^{-7} PE
6. Add 40µL of 10^{-3} PE; Final concentration: $3x10^{-7}$ PE
7. Add 140µL of 10^{-3} PE; Final concentration: 10^{-6} PE
8. Add 40µL of 10^{-2} PE; Final concentration: $3x10^{-6}$ PE
9. Add 140µL of 10^{-2} PE; Final concentration: 10^{-5} PE

Acetylcholine (ACh) Concentration Response Curve

Preparation of ACh serial dilution: Add 900µL of APSS to five 1ml tubes. Add 100µL of 1M stock ACh to the first tube; final concentration 10^{-1} ACh. Vortex the tube, and add 100µL of the 10^{-1} ACh to the second tube; final concentration 10^{-2}

ACh. Vortex the second tube, and repeat pervious steps until 10^{-5} ACh concentration tube is mixed.

Add the following concentrations of ACh following 50% resting inner diameter preconstriction with PE. Record ID after each addition.

1. Add 20µL of 10^{-5} ACh; Final concentration: 10^{-9} ACh
2. Add 40µL of 10^{-5} ACh; Final concentration: $3x10^{-9}$ ACh
3. Add 140µL of 10^{-5} ACh; Final concentration: 10^{-8} ACh
4. Add 40µL of 10^{-4} ACh; Final concentration: $3x10^{-8}$ ACh
5. Add 140µL of 10^{-4} ACh; Final concentration: 10^{-7} ACh
6. Add 40µL of 10^{-3} ACh; Final concentration: $3x10^{-7}$ ACh
7. Add 140µL of 10^{-3} ACh; Final concentration: 10^{-6} PE
8. Add 40µL of 10^{-2} ACh; Final concentration: $3x10^{-6}$ Ach
9. Add 140µL of 10^{-2} ACh; Final concentration: 10^{-5} ACh

APPENDIX C

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

Institutional Animal Care and Use Committee (IACUC)
Office of Research Integrity and Assurance
Arizona State University
660 South Mill Avenue, Suite 315
Tempe, Arizona 85287-6111
Phone: (480) 965-4387 *FAX:* (480) 965-7772

Animal Protocol Review

ASU Protocol Number: **12-1233R**
Protocol Title: **Regulation of Vascular Reactivity in Birds**
Principal Investigator: **Karen Sweazea**
Date of Action: **12/21/2011**

The animal protocol review was considered by the Committee and the following decisions were made:

- ☐ The original protocol was APPROVED as presented.
- ☒ The revised protocol was APPROVED as presented.
- ☐ The protocol was APPROVED with RESTRICTIONS or CHANGES as noted below. The project can only be pursued, subject to your acceptance of these restriction or changes. If you are not agreeable, contact the IACUC Chairperson immediately.
- ☐ The Committee requests CLARIFICATIONS or CHANGES in the protocol as described in the attached memorandum. The protocol will be considered when these issues are clarified and the revised protocol is submitted.
- ☐ The protocol was approved, subject to the approval of a WAIVER of provisions of NIH policy as noted below. Waivers require written approval from the granting agencies.
- ☐ The protocol was DISAPPROVED for reasons outlined in the attached memorandum.
- ☐ The Committee requests you to contact _____ to discuss this proposal.
- ☐ A copy of this correspondence has been sent to the Vice President for Research.
- ☐ Amendment was approved as presented.

Documentation of Level III Training will need to be provided to the IACUC office before the participant can perform procedures independently. For more information on Level III requirements see https://researchintegrity.asu.edu/training/animals/levelthree

Total # of Animals: **240** **Pain Level:** **D** **Species:** **Birds**
Approval Period: **12/21/2011 – 12/20/2014**

Signature: C. Miller for D. Murphy Date: 12/21/11
IACUC Chair or Designee

Original: Principal Investigator
Cc: IACUC Office
IACUC Chair