Comparative Biochemistry and Physiology, Part A 201 (2016) 141–145

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

High glucose impairs acetylcholine-mediated vasodilation in isolated arteries from Mourning doves (*Z. macroura*)

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ARTICLE INFO

Article history:
Received 16 July 2013
Received in revised form 7 July 2016
Accepted 10 July 2016
Available online 19 July 2016

Keywords:
Avian
Artery
Hyperglycemia
Vasodilation
Superoxide
Acetylcholine

ABSTRACT

Normal avian plasma glucose levels are 1.5–2 times greater than mammals of similar size. In mammals, hyperglycemia induces oxidative stress and impaired endothelium-dependent vasodilation. Prior work has shown that mourning doves have high levels of antioxidants and isolated vessels have low endogenous oxidative stress. 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) were cannulated and pressurized in a vessel chamber then incubated with either normal or high glucose (20 mM vs. 30 mM) for 1 h 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 vasodilation 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 20 mM D-glucose and 10 mM L-glucose was not different from controls ($p = 0.273$). Rather, the impaired vasodilation was attributed to oxidative stress since superoxide levels were elevated 168 ± 42% ($p = 0.02$) and pre-exposure of arteries to the superoxide dismutase mimetic tiron (10 mM) improved vasodilation ($p < 0.05$). Therefore, isolated arteries from doves do not have endogenous mechanisms to prevent impaired vasodilation resulting from high glucose-mediated increases in oxidative stress.

1. Introduction

Endothelial dysfunction of the microvascular system is a major complication of diabetes and a predictor of future cardiovascular disease (de Haan and 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 endothelium-dependent hyperpolarization pathways (Tsai et al., 2011). This latter pathway induces vasodilation through diffusion of endothelial-derived hyperpolarizing factors (EDHFs) such as epoxyeicosatrienoic acids (EETs) to neighboring vascular smooth muscle cells where they induce hyperpolarization through activation of potassium channels. Evidence also suggests that the hyperpolarizing current can spread to the vascular smooth muscles cells through myoendothelial gap junctions (Ellinsworth et al., 2014; Garland and Dora, 2016).

In mammals, chronic and acute hyperglycemia increase the production of reactive oxygen species (ROS), resulting in the development of oxidative stress and endothelial dysfunction (de Haan and Cooper, 2011; Liu and Gutterman, 2002; Torimoto et al., 2013). 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 et al., 2010). In a hyperglycemic state, superoxide ($O_2^{\bullet -}$) production from mitochondrial respiration becomes elevated. ROS-induced vascular dysfunction occurs through impairment of endothelial-derived relaxing factors such as NO as well as EDHFs (Fatehi-Hassanabad et al., 2010; Potenza et al., 2009). Because $O_2^{\bullet -}$ 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 pro-inflammatory state (Inoguchi et al., 2003; Kojda and Harrison, 1999; Yao and Brownlee, 2010). EDHF-mediated dilation has been described as a compensatory mechanism to induce vasodilation when NO activity is impaired (Ozkan and Uma, 2005). However, EDHF-mediated relaxation is also diminished in the presence of high glucose. This can occur through reduced cytochrome p450 epoxygenase activity, which subsequently reduces endothelial

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http://dx.doi.org/10.1016/j.cbpa.2016.07.010
1095-6433/

production of EETs (Tsai et al., 2011). Downregulation of various potassium channels are also implicated in causing impaired endothelium-dependent hyperpolarization-mediated vasodilation resulting from hyperglycemia (Liu and Gutterman, 2002; Morales-Cano et al., 2015; Nieves-Cintrón et al., 2015; Wang et al., 2010).

Acetylcholine (ACh) is commonly used to assess endothelium-dependent vasodilation (Schuurman and Villamor, 2010; Taylor et al., 1995). ACh activates vasodilation in arteries through stimulation of NO, prostacyclin and endothelium-dependent hyperpolarization 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 not rely on either the NO or cyclooxygenase-dependent pathways (Jarrett et al., 2013). Rather, the main contributors to ACh-vasodilation is endothelium-dependent hyperpolarization through the activation of potassium channels (Jarrett et al., 2013).

The use of an avian model is a novel approach to study the pathways implicated in acute hyperglycemic conditions, because avian plasma glucose concentrations are normally 1.5–2 times greater than mammals of similar body mass (Braun and Sweazea, 2008). In fact, levels for wild mourning doves are 18.9 ± 2.9 mM (Range: 15–24 mM; Smith et al., 2011). In addition, a study of mourning doves held in captivity for 30 weeks measured reference glucose concentrations ranging from 19 to 40 mM (Schulz et al., 2000). Despite these naturally high avian glucose concentrations, studies have shown that mitochondria isolated from brain, heart and kidney tissues from pigeons produce significantly less $O_2^{\bullet -}$ and H_2O_2 compared to the same tissues isolated from rats (Ku and Sohal, 1993). Moreover, prior studies from mourning doves have shown that these birds have very high antioxidant capacities as well as circulating vitamins A and E, and uric acid concentrations in addition to low levels of oxidative stress within isolated arteries (Smith et al., 2011). However, it is unknown whether isolated avian arteries have endogenous mechanisms to prevent oxidative stress. Therefore, the hypothesis of the current study was that endothelium-dependent vasodilation of isolated avian arteries would not be impaired following acute exposure to high glucose.

2. Materials and methods

2.1. Animal model

Adult male and female mourning doves (*Z. macroura*; 119.6 ± 1.84 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 (Jarrett et al., 2013; Smith et al., 2011; 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.

2.2. 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 (~1 mm length; 123.4 ± 3.39 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 containing either 20 mM (control) or 30 mM glucose, 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 mm Hg with a servo-controlled peristaltic pump (Living Systems Instrumentation, Burlington, VT). The exterior of 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 was aerated to maintain pH and heated to 41 °C (normal dove body temperature).

2.3. Endothelium-dependent vasodilation

After equilibration for 30 min in APSS, isolated c. tibial arteries were superfused for 1 h with APSS containing either 20 mM or 30 mM glucose in the presence and absence of the superoxide dismutase mimetic 4,5-dihydroxy-1,3-benzene-disulfonic acid disodium salt (tiron; 10 mM). The 20 mM glucose solution was chosen as the control, because it is similar to normal physiological plasma glucose concentrations measured for mourning doves (17–20 mM) (Braun and Sweazea, 2008; Smith et al., 2011). According to the World Health Organization (2006), normal plasma glucose concentrations in humans are <5.6 mM, whereas levels of 6.1–6.9 mM are diagnostic of impaired fasting glucose and ≥7.0 mM is diagnostic of diabetes. The transition from normoglycemic to diabetes represents only a 25% increase in glucose concentrations (WHO, 2006). In fact, increases in plasma glucose concentrations over 50% of normoglycemic are often measured (>9 mM) in individuals with diabetes (Torimoto et al., 2013). Thus, since birds naturally have high circulating glucose concentrations (Braun and Sweazea, 2008), we chose to determine the effects of a 50% increase in glucose from 20 mM (normal) to 30 mM (high).

Vessels were pre-constricted to 50% of their resting inner diameter with increasing concentrations of phenylephrine (10^{-8} to 10^{-5} M, 5 min each step) in the superfusate followed by exposure to stepwise increases of acetylcholine (ACh; 10^{-8} to 10^{-5} M, 5 min each step). To measure the contribution of increased osmolarity to vasoreactivity, separate arteries were pre-exposed to APSS containing either 20 mM glucose and 10 mM D-mannitol or 20 mM glucose and 10 mM L-glucose. D-Mannitol and L-glucose are both used as osmotic controls in studies examining the effects of high glucose as they are not taken up by cells (El-Remessy et al., 2003). A limitation of mannitol is that it can exhibit antioxidant activity, whereas L-glucose does not have this effect (Karasu, 2000). 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 min 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 vasodilation was calculated.

2.4. Measurement of superoxide production

Vascular superoxide in c. tibial arteries was assessed using dihydroethidium (DHE) microfluorography. In the presence of superoxide, DHE oxidizes to form the fluorophore ethidium bromide (EtBr), which intercalates with DNA becoming trapped in the nuclei. A modified protocol from Bagi et al. (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 20 mM or 30 mM glucose and incubated for 60 min 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 min. Following a 5-min 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.