Cerebral Myogenic Reactivity and Blood Flow in Type 2 Diabetic Rats: Role of Peroxynitrite in Hypoxia-mediated Loss of Myogenic Tone

Aisha I. Kelly-Cobbs, Roshini Prakash, Maha Coucha, Robert A. Knight, Weiguo Li, Safia N. Ogbi, Maribeth Johnson, Adviye Ergul

From the Charlie Norwood Veterans Administration Medical Center (W.L., A.E.), Departments of Physiology (A.I.K, M.C., S.N.O., W.L., A.E.) and Biostatistics (M.J.), Georgia Health Sciences University, Program in Clinical and Experimental Therapeutics, University of Georgia (R.P., A.E.), Augusta, Georgia and Department of Neurology – NMR Research, Henry Ford Health System (R.A.K), Detroit, Michigan
Running Title: Cerebrovascular reactivity and oxidative stress in diabetes

Address reprint requests to

Adviye Ergul, M.D., Ph.D.
Georgia Health Sciences University
Department of Physiology
1120 15th Street CA2094
Augusta, GA 30912
Ph: 706-721-9103
Fax: 706-721-7299
Email: aergul@georgiahealth.edu

Number of text pages: 21
Number of tables: 1
Number of figures: 5
Number of references: 60
Number of words in Abstract: 250
Number of words in Introduction: 896
Number of words in Discussion: 1,648
Recommended section assignment: Endocrine and Diabetes

List of non-standard abbreviations: FeTPPs (5,10,15,20-Tetrakis(4-sulfonatophenyl)prophyrinato iron (III), Chloride)
Abstract

Dysregulation of cerebral vascular function and ultimately cerebral blood flow (CBF) may contribute to complications such as stroke and cognitive decline in diabetes. We hypothesized that: 1) diabetes-mediated neurovascular and myogenic dysfunction impairs CBF, and 2) under hypoxic conditions cerebral vessels from diabetic rats lose myogenic properties due to peroxynitrite (ONOO•)-mediated nitration of vascular smooth muscle (VSM) actin. Functional hyperemia, the ability of blood vessels to dilate upon neuronal stimulation, and myogenic tone of isolated middle cerebral arteries (MCA) were assessed as indices of neurovascular and myogenic function, respectively, in 10-12-week control and type 2 diabetic Goto-Kakizaki rats. In addition, myogenic behavior of MCAs, nitrotyrosine (NY) levels, and VSM actin content were measured under normoxic and hypoxic (oxygen glucose deprivation-OGD) conditions with and without ONOO• decomposition catalyst, FeTPPs. Percent (%) myogenic tone was higher in diabetes and forced dilation occurred at higher pressures. Functional hyperemia was impaired. Consistent with these findings, baseline CBF was lower in diabetes. OGD reduced % myogenic tone in both groups and FeTPPs restored it only in diabetes. OGD increased VSM NY in both groups and while FeTPPs restored basal levels, it did not correct reduced filamentous/globular (F/G) actin ratio. Acute alterations in VSM ONOO• levels may contribute to hypoxic myogenic dysfunction but this cannot be solely explained by decreased F/G actin ratio due to actin nitration and mechanisms may differ between control and diabetic animals. Our findings also demonstrate that diabetes alters the ability of cerebral vessels to regulate CBF under basal and hypoxic conditions.
Introduction

Type 2 diabetes (T2D) promotes widespread morphological and functional aberrations within the cardiovascular system, including the cerebral vessels (Mayhan, 1993). As a consequence, impairment of intrinsic mechanisms that safeguard the brain against disruptions in blood flow increase the risk of developing micro- and macrovascular complications such as acute ischemic stroke (Reusch, 2003; Fowler, 2008) and cognitive decline (Carlsson, 2010; Humpel, 2011). The brain is composed of highly metabolic neural tissue that is completely dependent upon sufficient blood flow for providing nutrients and clearing metabolic wastes (Drake and Iadecola, 2007). The importance of adequate cerebral perfusion is underscored by the fact that under normal conditions: 1) neural and metabolic cues ensure proper distribution of blood flow; 2) autoregulation protects the brain against fluctuations in the systemic circulation (Johnson, 1964); and 3) neuronal activity is tightly coupled to blood flow demand (functional hyperemia) (Iadecola, 2004; Girouard and Iadecola, 2006). Reduced cerebral blood flow (CBF) has been described in diabetic patients as well as in animal models of type 1 diabetes (T1D) (Duckrow et al., 1987; McCall, 1992) which may contribute to the progression of neurodegenerative disorders (Iadecola, 2004; Ohara et al., 2011) and complicate ischemia/reperfusion injury in diabetes (Hasselbalch et al., 2001; Li et al., 2009). The basal tension within the cerebral vascular smooth muscle (VSM), termed “myogenic tone”, permits dynamic flow changes in response to the energetic demands of the neuronal tissue and is an important modulator of CBF autoregulation (Osol et al., 1991; Cipolla et al., 1997a). In the brain, large arteries such as the middle cerebral arteries (MCAs) contribute 20-30% of the total cerebrovascular resistance (Faraci and Heistad, 1990). Zimmermann et al. demonstrated that MCAs isolated from streptozotocin (STZ)-induced diabetic rats exhibit enhanced constriction (Zimmermann et al., 1997). Similarly, Jarajapu et al.
reported altered myogenic reactivity and increased tone in posterior cerebral arteries (PCAs) from type 2 diabetic BBZDR/Wor rats (Jarajapu et al., 2008). However, the effect of diabetes on functional hyperemia, myogenic tone and CBF in a T2D model that mimics blood glucose levels seen in patients remains to be determined.

The autoregulatory efficiency of cerebral vessels is dependent upon polymerization and depolymerization of VSM actin filaments in response to changes in transmural pressure across the vessel (Cipolla and Osol, 1998). The cytoskeletal machinery is particularly vulnerable to oxidative stress (Maneen et al., 2006; Maneen and Cipolla, 2007). Peroxynitrite (ONOO⁻), a stable reactive oxygen and nitrogen species, is capable of dismantling filamentous (F) actin and preventing globular (G) actin polymerization (Clements et al., 2003; Tiago et al., 2006). Together, these events can lead to an impaired VSM myogenic response (Cipolla et al., 2001; Maneen et al., 2006; Maneen and Cipolla, 2007). Loss of myogenic activity is particularly detrimental during focal cerebral where it can contribute to perturbation of vascular integrity and ensuing hemorrhage and edema (Maneen et al., 2006; Dohmen et al., 2007; Maneen and Cipolla, 2007). Previous studies have reported diminished myogenic tone in PCAs isolated from normoglycemic rats following transient middle cerebral artery occlusion (tMCAO) (Cipolla et al., 2001; Maneen et al., 2006; Maneen and Cipolla, 2007). Furthermore, this loss of tone was correlated with an ONOO⁻-mediated decrease in VSM F actin. Due to the potential mechanical injury sustained from tMCAO beyond 30 min (Cipolla et al., 2001), however, there are few studies examining the effects of ischemia/reperfusion injury on MCA myogenic reactivity. Furthermore, the mechanisms underlying the impairment of myogenic reactivity observed in diabetes, particularly under hypoxic conditions, are not fully understood. Thus, the purpose of our study was to use an ex vivo oxygen glucose deprivation (OGD) protocol that would enable us
to test the hypothesis that basal cerebrovascular myogenic tone is increased in the Goto-Kakizaki (GK) rat model of T2D diabetes and that following OGD, cerebral vessels from these chronically diabetic rats will have markedly reduced myogenic reactivity due to ONOO–mediated nitration of the VSM actin cytoskeleton.

Materials and methods

Animals

All experiments were performed on 10- to 12-week-old male Wistar (n=31) rats (Harlan; Indianapolis, ID) and age-matched diabetic GK (n=33) rats (In-house bred, derived from the Tampa colony). This age range was based on our previous studies which showed greater vascular injury, hemorrhage and edema, in this model at this age after ischemia/reperfusion injury of the brain. Glucose intolerance can be observed as early as 2 weeks of age in the GK rat and onset of moderate hyperglycemia can be as early as 5-6 weeks of age. During weeks 6-12, plasma insulin levels are elevated and then decrease to levels lower that observed in Wistar rats (Goto et al., 1976; Harris et al., 2005). This non-obese model of type 2 diabetes is ideal for these studies because diabetes-induced cerebrovascular alterations can be studied in the absence of confounding comorbidities such as hypertension or hyperlipidemia (Harris et al., 2005; Elgebaly et al., 2008). The animals were housed at the Georgia Health Sciences University animal care facility that is approved by the American Association for Accreditation of Laboratory Animal Care. All protocols were approved by the institutional animal care and use committee. Animals were fed standard rat chow and tap water ad libitum. Body weights and blood glucose measurements were taken biweekly. Blood glucose measurements were taken from tail vein samples using a commercially available glucometer (Freestyle, Abbott Diabetes Care, Inc;
Mean arterial pressure (MAP, mmHg) was measured using the tail-cuff method. All animals were anesthetized with pentobarbital sodium (Fatal-Plus, Vortech Pharmaceuticals Ltd; Dearborn, MI), exsanguinated via cardiac puncture, and decapitated to extract the brain.

**Measurement of functional hyperemia**

Functional hyperemia was assessed two days prior to ischemic injury by measuring the changes in CBF in the somatosensory cortex upon whisker stimulation. For this purpose, trimmed contralateral whiskers were gently stroked at a frequency of 2.5 Hz using a cotton tip attached to a vortex (Kaarisalo et al., 2005; Nicolakakis et al., 2008). A PIM 3 laser Doppler flowmeter (Perimed Systems; Stockholm, Sweden) was programmed to scan an area covering somatosensory cortex which is supplied by the MCA. Changes in CBF were expressed as % increase relative to resting levels. The laser beam was directed at the skull surface (2 mm posterior and 5 mm lateral to the bregma) by a moving-mirror system in the scanner without tissue contact. In this system, a built-in photo detector detects the reflected light from moving blood cells within 0.5 cm of the cortical surface and a color coded image is acquired based on the concentration and mean velocity of these blood cells using the LDPIwin software (Perimed, North Royalton, OH).

**Measurement of CBF by Magnetic Resonance Imaging**

All MRI measurements of CBF were acquired using a 7 Tesla 20 cm bore magnet interfaced to a Bruker console. The animals were initially anesthetized using 1.0-2.0% isoflurane in a 2:1 N₂O:O₂ gas mixture administered by spontaneous respiration. After induction, the animals were placed on a plastic platform equipped with ear bars to stabilize the head and a nose cone for administration of gases. The platform then fits inside a 5 cm diameter quadrature
transmit/receive birdcage coil tuned to the resonant frequency of water (300 MHz). CBF measures were obtained in cerebral tissue using an arterial spin labeling (ASL) technique (Detre et al., 1992; Williams et al., 1992). Adiabatic inversion of inflowing arterial water proton (Dixon et al., 1986) was accomplished via an axial gradient of ±0.3 kHz/mm, and a one-second continuous wave (CW) radio-frequency (RF) power of approximately 0.3 kHz at a frequency offset of ±6 kHz. This was followed by a spin-echo imaging sequence with repetition time/echo time = 1000ms/20ms. As indicated four preparations of the image were acquired with the gradient polarities and frequency offsets switched to remove the gradient asymmetry in the axial direction. The labeled slice is 2 cm distal from the imaging slice with 1 mm thickness. The field of view was 32 mm x 32 mm with a 64 x 64 matrix size. While this study did not employ MCA occlusion to induce ischemia, the regions of interest (ROI) for CBF measurements were based on regions where brain infarcts occur if the MCA is occluded and included entire parietal cortex or striatum (Knight et al., 1998).

MCA function and morphometry

MCAs were quickly excised and used within 45 minutes of isolation to ensure viability of the vessels. A pressure arteriograph system (Living Systems; Burlington, VT) was used to evaluate MCA structure, myogenic tone, and mechanical properties. For these studies, MCA segments approximately 200-250 μm in diameter and proximal to the junction between the MCA and the inferior cerebral vein were used exclusively. The vessels were first mounted onto glass cannulas in an arteriograph chamber and HEPES bicarbonate buffer (in mM: 130 NaCl, 4 KCl, 1.2 MgSO₄, 4 NaHCO₃, 10 HEPES, 1.18 KH₂PO₄, 5.5 glucose, 1.8 CaCl₂) was circulated and maintained at 37±0.5°C. The MCA segments were then pressurized at 20 mmHg for 1 h to generate spontaneous tone. A video dimension analyzer connected to the arteriograph system
was used to measure media thickness (MT) and lumen diameter (LD) at pressures ranging from 0-180 mmHg, in 20 mmHg increments. The first measurement was taken at 5 mmHg because negative pressure is generated at 0 mmHg, causing the vessel to collapse. All vessels were exposed to each pressure point for 5 min before readings were recorded. Pressure-diameter curves were obtained, first in the presence of Ca\(^{2+}\) to observe the vessels’ contractile properties, and then in Ca\(^{2+}\)-free buffer with the addition of 10\(^{-7}\) M papaverine hydrochloride to evaluate the vessels’ passive properties.

**Data calculations**

Using the MT and LD measurements obtained in active conditions (in the presence of Ca\(^{2+}\)) and in passive conditions (in the absence of Ca\(^{2+}\)), the following parameters related to MCA structure, myogenic tone, and mechanical properties can be calculated: 

- **Media Thickness** (MT, \(\mu\text{m}\)) = Left Wall + Right Wall;
- **Outer Diameter** (OD, \(\mu\text{m}\)) = LD + MT;
- **Medial/Lumen ratio** (M/L) = MT/LD, and
- **Percent Myogenic Tone** (% tone) = 1 - (Active OD/Passive OD) x 100.

**Ex vivo oxygen-glucose deprivation**

A modified ex vivo oxygen-glucose deprivation protocol (Lynch et al., 2006) was used to determine the effects of OGD on MCA structure, myogenic tone, and mechanical properties. Pressurized MCA segments approximately 250 \(\mu\text{m}\) in diameter were first equilibrated at 20 mmHg for 1 h in HEPES buffer containing 5.5 mM glucose to allow the development of spontaneous tone. Immediately after the equilibration period, induction of OGD was performed by sealing off the arteriograph chamber and connecting it to a gas line supplying 95% N\(_2\)/5% CO\(_2\) at a rate of 20 ml/min while low glucose (1 mM) HEPES buffer, also gassed with the N\(_2\)/CO\(_2\) mixture, was circulated and maintained at 37±0.5°C. The MCAs were exposed to OGD...
for 20 min before they were returned to normoxic conditions and buffer containing 5.5 mM glucose was re-circulated for the remainder of the experiment. Pressure-diameter curves were obtained as described above.

**Acute treatment with FeTPPs or peroxynitrite**

To determine the effects of acute antioxidant treatment on vascular structure and function, vessels were equilibrated for 1 h, subjected to 20 min of OGD exposure as described in the previous section, and then the peroxynitrite decomposition catalyst, 5,10,15,20-Tetrakis(4-sulfonatophenyl) proprhibrinato iron (III), Chloride (FetPPs (Sharma et al., 2007), 2.5µM (Abdelsaid et al., 2010), Calbiochem; San Diego, CA) was added to the recirculating HEPES buffer immediately following OGD treatment and allowed to circulate for the remainder of the experiment. To further investigate the effect of peroxynitrite on control and diabetic vessels, MCAs isolated from right and left side of the same animal were treated with either vehicle or peroxynitrite (10 µM) in the arteriograph chamber after the equilibration period and then pressure curves were obtained.

**Detection of hypoxia**

To confirm hypoxia (pO₂ < 10 mmHg) in MCAs exposed to OGD, whole MCA segments were treated with 200 µM pimonidazole hydrochloride (Natural Pharmacia International, Inc.; Burlington, MA). The MCA segments were first equilibrated at 20 mmHg for 1 h then pressure-fixed in 4% paraformaldehyde in PBS following OGD exposure. A Hypoxyprobe-1 Omni Kit (NPI, Catalog #HPI-100) was used to probe for pimonidazole hydrochloride adducts using conventional immunohistochemical methods. Images of stained vessels (40x, oil) were captured
using a Zeiss inverted confocal microscope (LSM 510; Carl Zeiss Microimaging, LLC; Thornwood, NY) and processed using Zen 2008 software.

*F and G actin staining and quantification*

The MCA segments were pressure-fixed in methanol-free 4% paraformaldehyde in PBS for 1 h at 20 mmHg. Whole-mounted tissues were then stained with Oregon Green 488-conjugated phalloidin fluorescent probe (Molecular Probes, Inc.; Eugene, OR) and Alexa 594-conjugated DNase I (Molecular Probes, Inc.) to identify F and G actin cytoskeletal proteins, respectively. Stained vessels were opened lengthwise by a longitudinal cut with microsurgical scissors (Fine Science Tools; Foster City, CA) and mounted for imaging. Z-stacks (8 µm; 0.5 µm intervals) of the VSM layer were captured at 63x using an LSM 510 inverted confocal microscope and processed using Zen 2008 software. This region of interest was confirmed by staining for nuclei in a separate set of vessels and observing their distinctive parallel arrangement. Image J software (NIH; Bethesda, MD) was used to quantify F and G actin volume intensity.

*Nitrotyrosine quantification*

Nitrotyrosine levels were used as an indirect marker of ONOO⁻ generation in blood plasma and MCAs. Snap-frozen MCAs were homogenized as previously described (Harris et al., 2005; Portik-Dobos et al., 2006; Elgebaly et al., 2007). Total nitrotyrosine levels were determined via slot blot analysis using an anti-nitrotyrosine monoclonal antibody (Millipore; Lake Placid, NY).

*Statistical Analysis*

Data are expressed as mean ± SE. Data were evaluated for normality and appropriate transformations were used when necessary. A rank transformation was used for MCA
nitrotyrosine levels prior to analysis. Area under the curve (AUC) across intraluminal pressure (60 mmHg to 160 mmHg) was calculated using NCSS 2007 (NCSS, LLC, Kaysville, UT). A series of analyses were used to assess the effect of disease and treatment. A 2 disease (control vs. diabetes) by 2 OGD (no vs. yes) ANOVA was used to assess the effect of hypoxia on diseased vessels. A 2 disease+OGD (control vs. diabetes) by 2 FeTPPs (no vs. yes) ANOVA was used to assess the effect of FeTPPs treatment on disease vessels after OGD exposure. An interaction would indicate a differential effect of hypoxia or FeTPPs treatment on diabetic vessels. A Tukey’s test was used to adjust for the multiple comparisons used to assess significant effects. Statistical significance was determined at alpha=0.05. SAS® version 9.2 was used for all analyses (SAS Institute, Inc., Cary, NC).

Results

Metabolic parameters

Metabolic parameters for experimental groups are summarized in Table 1. GK rats are a lean model of T2D. These spontaneously diabetic animals were smaller than their age-matched controls and beginning at 6 weeks of age displayed moderate hyperglycemia, as previously reported (Harris et al., 2005; Elgebaly et al., 2008). Blood pressure was slightly higher in diabetic animals compared to controls; however, these animals were not hypertensive.

CBF and functional hyperemia

Basal CBF measurements yielded 2 important findings. First, CBF in parietal/insular cortex and striatum was significantly lower in diabetic animals compared to controls (Fig. 1A). Second,
cortical CBF was greater than that measured in the striatum of control animals, but this difference was not observed in diabetic animals. In addition, relative increases in CBF upon stimulation of somatosensory cortex were lower in diabetic animals indicating impaired functional hyperemia and neurovascular coupling (Fig. 1B and C).

**MCA myogenic tone and reactivity**

Increasing intraluminal pressures from 80 to 180 mmHg generated greater myogenic tone in isolated MCAs from chronically diabetic rats, compared to their age-matched controls (Fig. 2A). Short-term OGD exposure diminished MCA myogenic response to increases in pressure from 20 to 180 mmHg (Fig. 2B). This reduced myogenic reactivity is particularly evident in MCAs from diabetic rats. The addition of FeTPPs following OGD treatment restored basal myogenic tone in vessels from diabetic rats, while control vessels were not affected (Fig. 2C).

Active pressure-diameter curves indicate that MCAs from diabetic rats constrict more in response to increases in pressure and forced dilatation occurs at higher pressures (Fig. 2D). Active lumen diameters were increased in all MCAs exposed to ex vivo OGD, however they were still capable of generating a blunted autoregulatory response (Fig. 2E). Acute FeTPPs treatment did reverse the effects of OGD on active lumen diameters in diabetic but not control group (Fig. 2F). To further confirm that peroxynitrite impacts myogenic reactivity and tone to a greater extent in diabetes, MCAs isolated from control or diabetic animals were treated with vehicle or peroxynitrite under normoxic conditions. While the myogenic tone of MCAs from control animals did not change in the presence of peroxynitrite, vessels from diabetic animals displayed a 39.4 ± 1.6 and 69.3 ± 16.9 % loss in myogenic tone at 100 and 140 mm-Hg.
pressures, respectively, supporting our hypothesis that cerebral vessels are highly sensitive to peroxynitrite in diabetes.

Confocal imaging results confirmed that hypoxia was detected only in MCAs incubated in pimonidazole hydrochloride at the time of OGD exposure. The presence of pimonidazole hydrochloride or OGD alone was not sufficient to detect hypoxia in these vessels (Fig. 2 inset).

**MCA structure**

After six weeks of moderate hyperglycemia exposure, MCA media thickness is indistinguishable from control vessels (Fig 3A). Arteriograph results also indicated MT was increased in all vessels exposed to OGD (Fig 3B). OGD-mediated media thickening was not reversed in control vessels following FeTPPs treatment (Fig. 3C).

Passive dilatation obtained under calcium-free conditions was similar to normoglycemic controls over the entire range of intraluminal pressures (Fig. 3D). Passive lumen diameters did not change after OGD treatment (Fig. 3E) or after peroxynitrite scavenging with FeTPPs (Fig. 3F).

Media-to-lumen ratios observed in MCAs from diabetic rats were similar to those of control vessels at all pressure points (Fig. 3G). At pressures beyond 40 mmHg, OGD exposure resulted in higher media-to-lumen ratios in both control and diabetic vessels (Fig. 3H). FeTPPs treatment had no effect on the media-to-lumen ratios from either OGD-treated group (Fig. 3I).

**F and G actin quantification**

Using confocal imaging, the VSM layer was identified by its distinctive parallel arrangement of cells and Z-stacks of this region of interest were captured and processed (Fig. 4A). In untreated vessels F/G actin ratios were similar. Furthermore, OGD exposure irreversibly reduced F/G ratios in both control and diabetic vessels. FeTPPs treatment did not have any effect in either...
group (Fig. 4B). Nitrotyrosine staining was more pronounced in both control and diabetic vessels after OGD exposure and this was reduced by FeTPPS treatment (Fig. 4C). In these samples, there was colocalization of G actin and nitrotyrosine staining suggesting that nitration contributes to the increases in the G actin pool.

*MC* _a peroxynitrite generation_

Nitrotyrosine quantification was used as an indirect measurement of basal ONOO⁻ generation in plasma and isolated MCAs from control and diabetic animals. After six weeks of diabetes, nitrotyrosine levels in plasma (Fig. 5A) and untreated whole vessel homogenates (Fig. 5B) were double that of controls. Nitrotyrosine levels were elevated in whole MCA homogenates from OGD-treated control vessels, whereas OGD exposure did not raise nitrotyrosine levels beyond baseline values that are already high in MCAs from diabetic rats. Similarly, acute treatment with FeTPPS restored basal nitrotyrosine levels in OGD-treated control vessels but no significant difference was detected in MCAs obtained from diabetic animals.

**Discussion**

The objective of the current study was to address the following questions: 1) What structural and functional changes occur in cerebral vessels as a result of chronic diabetes exposure and how do they affect cerebral blood flow?; 2) Do cerebral vessels from control and diabetic animals respond similarly to acute OGD?; and 3) Are the effects of OGD mediated by peroxynitrite? Our results indicate that chronic diabetes exposure results in dysregulation of cerebral blood flow that is accompanied by heightened myogenic reactivity and impaired neurovascular coupling. In the early stages of diabetes, however, pathological structural changes
are not present in large cerebral vessels. Short-term OGD blunts cerebrovascular autoregulation and attenuates myogenic responses, irrespective of glycemic status. The loss of myogenic reactivity and tone observed in vessels from diabetic rats is associated with peroxynitrite generation during OGD; however, these effects cannot be solely explained by peroxynitrite-mediated nitration of the VSM actin cytoskeletal filaments.

The brain relies completely upon continuous perfusion by cerebral vessels to ensure the normal health and function of nervous tissue; consequently, the cerebrovasculature is equipped to protect against dramatic fluctuations in blood flow. Dysregulation of blood flow and impairment of the regulatory mechanisms that ensure perfusion matches neuronal demand both contribute to the micro- and macrovascular complications observed in diabetes. In this study, we provide two lines of evidence: 1) functional hyperemia is impaired in diabetes, and 2) basal CBF is reduced in diabetic animals compared to controls. The neurovascular dysfunction observed in the current study may be of vascular or neuronal origin or both. It is believed that under physiological conditions, functional hyperemia is principally regulated by penetrating parenchymal arterioles, astrocytes, and interneurons (Lecrux and Hamel, 2011). Although penetration of the brain is limited by Laser Doppler imaging, whisker stimulation tests allow us to evaluate functional hyperemia by detecting changes in CBF on the surface of the brain. These upstream cortical vessels are innervated by the peripheral nervous system but are also retrogradely affected by changes that occur in deep penetrating arterioles. There is also evidence that oxidative stress contributes to impaired functional hyperemia in other disease models (Girouard and Iadecola, 2006). In our model of type 2 diabetes, we believe that oxidative stress may also be involved and further experiments are needed to directly address this possibility. The latter set of data is in accordance to previous reports that chronic diabetes exposure with
uncontrolled moderate hyperglycemia contributes to reduced CBF (Duckrow et al., 1987; Kaplar et al., 2009). There are experimental studies that have shown increased CBF in diabetes, but these have primarily been described in acute STZ-induced models of diabetes exhibiting severe hyperglycemia with plasma glucose levels greater than 300 mg/dL (Al-Saeedi, 2008). Finally, failure to detect differences in basal CBF or reports of higher CBF in diabetic patients likely results from a study population with good glycemic control (Neil et al., 1987).

Findings from functional studies regarding the effects of hyperglycemia on cerebrovascular reactivity and tone are also conflicted, stemming from differences in the derivation, duration, and severity of high glucose exposure. Cipolla et al. reported that endothelium-dependent nitric oxide (NO) and prostaglandin mechanisms contributed to reduced myogenic tone in posterior cerebral arteries (PCAs) isolated from normoglycemic rats in response to acute exposure to high glucose concentrations (Cipolla et al., 1997b); however, these vessels were acutely perfused with physiological buffers containing substantially high glucose levels upwards of 790 mg/dL. In this study, we give evidence that despite the absence of pathological remodeling, chronic moderate hyperglycemia contributes to increased myogenic tone and forced dilation at higher pressures in cerebral arteries such as the MCA. This data is in agreement with earlier observations that vasomotor dysfunction in diabetes precedes the advent of structural pathologies (Bagi et al., 2004; Oizumi et al., 2006). We also reported that metformin treatment started at the onset of diabetes prevents increased myogenic tone in this model suggesting that hyperglycemia mediates this effect (Elgebaly et al., 2010). Diabetic BBZDR/Wor rats used in the study by Jarajapu et al. had glucose levels in excess of 400 mg/dL; however, PCAs isolated from these animals exhibited increased tone after four weeks of diabetes resulting from increased phospholipase C activation (Jarajapu et al., 2008). These
vessels also displayed heightened vascular resistance as evidenced by forced dilation at higher pressures. Similarly, chronic studies by Zimmermann et al. demonstrated that MCAs isolated from STZ-induced diabetic rats exhibit enhanced constriction and myogenic tone attributed to hyperglycemia-mediated increases in membrane depolarization due to diminished responses to ATP-sensitive potassium channel openers (Zimmermann et al., 1997).

As the incidence of type 2 diabetes rises worldwide, there is an increasingly urgent need for effective therapies targeted to this stroke-prone population. However, it is not fully understood how diabetes-mediated changes in the cerebrovasculature contribute to ischemia-reperfusion injury. Our ex vivo OGD studies were conducted to see if we would observe similar results from earlier functional studies reported in cerebral vessels exposed to ischemia/reperfusion injury in normoglycemic animals that had undergone transient focal cerebral ischemia (Cipolla et al., 2001; Cipolla and Curry, 2002; Maneen et al., 2006; Maneen and Cipolla, 2007; Jimenez-Altayo et al., 2009; Perez-Asensio et al., 2010). Specifically, we were curious to see whether OGD-treated vessels isolated from moderately hyperglycemic rats would also exhibit loss of tone due to decreases in VSM F/G actin ratios and increases in lumen diameter and wall thickness. Consistent with aforementioned studies, we provide evidence that vessels from control animals display reduced tone associated with reduced F/G actin ratios. We also observed the protective hypertrophy and vasodilator responses that reduce wall stress in response to hypoxia (Jimenez-Altayo et al., 2009). Myogenic tone was also dramatically reduced in MCAs from diabetic animals and this was associated with decreased F/G actin ratio and increased media thickness.

Alone, hyperglycemia increases both superoxide (O$_2^-$) and NO levels, driving the production of the cytotoxic radical ONOO$^-$ while reducing NO bioavailability within the
vasculature (El-Remessy et al., 2010), however ONOO$^-$ is also generated during ischemia/reperfusion (Fukuyama et al., 1998; Gursoy-Ozdemir et al., 2004). Peroxynitrite is capable of thiol oxidation and tyrosine nitration, both of which can alter the function of enzymes and other cellular components (Fukuyama et al., 1998; Turko and Murad, 2002). Our present findings suggest that type 2 diabetic GK rats have greater plasma nitrotyrosine levels compared with normoglycemic controls, consistent with data published in humans (Turko and Murad, 2002). Furthermore, nitrotyrosine levels in whole MCA homogenates isolated from diabetic rats were double that of controls. In light of our functional data, we predicted that nitrotyrosine levels in MCAs exposed to \textit{ex vivo} OGD/reperfusion would be higher than isolated control vessels exposed to the same intervention. Furthermore, we anticipated that acute antioxidant treatment with FeTPPs would result in a greater reduction in the vessels isolated from diabetic animals. The data obtained, however, shows that nitrotyrosine levels are greater in are similar in whole MCAs from control and diabetic rats after OGD and FeTPPs reduces nitrotyrosine in both groups. When we examined the nitrotyrosine levels within the VSM of untreated MCAs using confocal microscopy, we found that levels were similar in control and diabetic rats at baseline and after OGD. Based on these findings we can deduce that in early diabetes the endothelium is the primary contributor of peroxynitrite generation and may impact potassium channels and endothelial nitric oxide synthase (eNOS) activity. There is evidence to suggest that calcium-activated endothelial potassium channels influence myogenic tone (Zimmermann, 1997 #4277) and any alterations in the activity of these targets may contribute to the greater cerebrovascular myogenic tone we observe in diabetic animals. Finally, previous studies have reported that reductions in myogenic tone after ischemic injury in otherwise healthy animals were associated with increased ONOO$^-$ and nitrotyrosine formation that colocalized with actin (Maneen et al.,
Our current study provides evidence that diminished myogenic tone in isolated vessels cannot be solely explained by tyrosine nitration of F actin since ONOO⁻ scavenging prevented nitrotyrosine formation but did not restore F/G actin ratios in control vessels. FeTPPs did not restore myogenic tone, either. These findings suggest that in control vessels, another mechanism may be contributing to the decrease in F/G actin ratio and accompanying loss of tone. In diabetes, however, FeTPPs does not prevent the decrease in F/G actin ratio but is able to restore tone implicating that there must be other targets of ONOO⁻ that contribute to the regulation of tone in diabetes, such as large calcium-activated potassium channels (BK channels) on VSMCs. Alternatively, ONOO⁻ might modify the actin cytoskeleton by another mechanism such as thiol oxidation.

There are several limitations to this study. While hypoxia was detected in the ex vivo preparation, we have not been able to determine whether the levels of hypoxia replicate those observed in ischemia/reperfusion injury in vivo. Secondly, based on previous studies we postulated that the effects observed in OGD would be mediated by ONOO⁻, however we cannot discount the contribution of other sources of oxidative and nitrosative stress on vascular reactivity, tone, and structure. Also, it is possible that the irreversible reduction in actin cytoskeleton polymerization observed in OGD-treated vessels results in oxidative stress-mediated glutathionylation of actin cysteiny1 residues or protein aggregation due to carbonylation and may not be the result of protein nitration, as previous hypothesized (Johansson and Lundberg, 2007; Dailianis et al., 2009). Lastly, the results obtained in vessels from diabetic animals cannot be explained solely by the effects of oxidative stress generated during hypoxic conditions. Hyperglycemia also promotes abnormal responses to vasoactive agents (Schwaninger et al., 2003; Didion et al., 2005). For example, we have reported that MCAs and
BAs from type 2 diabetic rats have heightened sensitivity to the vasoconstrictor endothelin-1 that is abolished by chronic endothelin receptor blockade (Harris et al., 2008). Thus, cerebral dysfunction in diabetes is multifactorial in nature and by uncovering these mechanisms we can implement better strategies to safeguard against vascular complications of the disease.
Author Contributions

Participated in research design: Ergul, Kelly-Cobbs, and Knight

Conducted experiments: Kelly-Cobbs, Coucha, Knight, Li, Ogbi, and Prakash

Performed data analysis: Ergul, Johnson, Kelly-Cobbs, and Knight

Wrote or contributed to the writing of the manuscript: Ergul, Johnson, Kelly-Cobbs, Knight, Li, and Prakash
References


Footnotes

The study was supported by in part by and award from the National Institute of Neurological Disorders And Stroke [F31NS066746] to Aisha Kelly-Cobbs. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Neurological Disorders And Stroke or the National Institutes of Health.

Adviye Ergul is a research pharmacologist at the Charlie Norwood Veterans Affairs Medical Center in Augusta, Georgia. Additional support was provided in part by Veteran’s Affairs Merit Award [BX000347]; National Institutes of Health award [NS054688]; and American Heart Association Established Investigator Award to [0740002N] to Dr. Ergul.

Figure Legends

Figure 1. CBF and functional hyperemia are compromised in diabetes. A representative color-coded image of CBF obtained by MRI is shown on the left (A). Quantitative analysis of readings in the parietal cortex or striatum (indicated by white circles) from all animals is given in the bar graphs on the right. Tracing of changes in CBF as measured by laser Doppler directed at 2mm posterior and 5 mm lateral to the bregma scanning during whisker stimulation in anesthetized animals (B). Average of the percent CBF change given in Panel B over the stimulation period (C). Abbreviations: CBF, cerebral blood flow. Results are given as mean ± SE, n=4-12, *p<0.05 vs. cortex, ***p<0.001 vs. control.

Figure 2. Effects of diabetes, OGD, and acute antioxidant treatment on myogenic responses in isolated MCAs over increasing intraluminal pressures. Myogenic tone at intraluminal pressures of 120 and 140 mmHg was increased in MCAs after 6 weeks of diabetes exposure, *p<0.05 vs. control (A). A 2 (control vs. diabetes) x 2 (untreated vs. OGD) analysis of data shown on panels A and B indicated that the OGD reduced tone and this was greater in diabetes, *p<0.05 vs. untreated or control + OGD (B). A 2 (untreated vs. OGD) x 2 (vehicle vs. FeTPPS) analysis of data shown on panels B and C indicated that FeTPPs (2.5 μM) reversed OGD-induced reduction in tone only in vessels from diabetic animals, ***p<0.001 vs. OGD (C). Though not significant, the MCAs from chronically diabetic rats demonstrated a rightward shift in the autoregulatory curve (D). Furthermore, OGD increased the active lumen diameters of MCAs (E) and acute antioxidant treatment with FeTPPs following OGD exposure restored basal levels only in diabetic animals, *p<0.05 vs. OGD (F). The presence of hypoxia during OGD exposure was confirmed with pimonidazole hydrochloride (inset). Grey areas denote the normal autoregulatory range (40-120 mmHg) for MCAs. Abbreviations: PIM, pimonidazole hydrochloride; OGD, oxygen-glucose deprivation. Results are given as mean ± SE, n=6-8.

Figure 3. Effects of diabetes, OGD, and acute antioxidant treatment on MCA structure. The MCA media thickness in early diabetes is similar to untreated control vessels (A). A 2 (control vs. diabetes) x 2 (untreated vs. OGD) analysis of data shown on panels A and B indicated that the exposure to OGD induced media thickening in MCAs in both groups, *p<0.05 vs. untreated (B). Peroxynitrite scavenging does not correct media thickening in OGD-treated control vessels, ***p<0.001 vs. OGD (C). Under calcium-free conditions, MCA lumen diameters were similar.
between untreated control and diabetic rats (D). There was no change in passive lumen
diameters of either control or diabetic MCAs after OGD exposure with (E) or without FeTPPs
(2.5 μM; F). Media-to-lumen ratios were similar in vessels from untreated control and diabetic
rats (G). Higher media-to-lumen ratios were observed after OGD-exposure (H). FeTPPs had no
effect on larger media-to-lumen ratios across the entire range of pressures in OGD-exposed
MCAs (I). Grey areas denote the normal autoregulatory range (40-120 mmHg) for MCAs.
Results are given as mean ± SE, n=6-8.

**Figure 4.** Effects of diabetes and hypoxia on VSM actin cytoskeleton and total protein nitration. Representative confocal microscopy images of MCA segments stained for F and G actin
cytoskeletal filaments and colocalization of actin filaments with nitrotyrosine (A). A 2 (control
vs. diabetes) x 2 (untreated vs. OGD) analysis of data shown indicated that OGD mediated a
decrease in F/G actin ratio in both groups, ***p<0.001 vs. untreated. FeTPPs (2.5 μM) did not
restore F/G actin ratio in either group (B). Vascular smooth muscle nitrotyrosine levels are
similar in untreated groups. OGD increased VSM nitrotyrosine levels in MCAs in both groups,
***p<0.001 vs. untreated. Acute treatment with FeTPPs after OGD exposure reversed this
effect, **p<0.05 vs. OGD (C). Abbreviations: C, control; D, diabetes; C + O, control + OGD; D
+ O, diabetes + OGD; C + O + F, control + OGD + FeTPPs; D + O + F, diabetes + OGD +
FeTPPs; F actin, filamentous actin; G actin, globular actin; VSM, vascular smooth muscle.
Results are given as mean ± SE, n=3-4.

**Figure 5.** Effect of diabetes on circulating and tissue (MCA) nitrotyrosine levels. Basal
nitrotyrosine (NY) levels are higher in plasma samples from chronically diabetic rats, **p<0.01
vs. control (A). A 2 (control vs. diabetes) x 2 (untreated vs. OGD) analysis of data indicated that
there is a disease and treatment interaction in response to OGD treatment in control vs. diabetic
vessels. OGD increases whole MCA nitrotyrosine levels in control but not in diabetes, **p<0.01
vs. untreated (B). Treatment with FeTPPs (2.5 μM) counteracts this response in controls without
any effect on diabetic vessels *p<0.05 vs. OGD. Abbreviations: MCA, middle cerebral artery;
VSM, vascular smooth muscle; IOD, integrated optical density. Results are given as mean ± SE,
n=3-8.
Table 1. Metabolic parameters of experimental animals. Results are given as mean ± SE, *p<0.05 vs. control, †††p<0.001 vs. control.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=31)</th>
<th>Diabetes (n=33)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>310 ± 2.6</td>
<td>296 ± 4.8*</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>97.5 ± 2.3</td>
<td>180.0 ± 8.2†††</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>102 ± 1</td>
<td>108 ± 5</td>
</tr>
</tbody>
</table>
Figure 4

A. F actin  G actin  Nitrotyrosine  Merged

C

D

C + O

D + O

C + O + F

D + O + F

B.

VSM F/G actin

Control (n=11)  Diabetes (n=5)

Untreated  OGD  OGD + FeTPPs

C.

VSM Nitrotyrosine Intensity

Control (n=9)  Diabetes (n=9)

Untreated  OGD  OGD + FeTPPs
Figure 5

A. Plasma Nitrotyrosine IOD (pixels)

B. Whole MCA Nitrotyrosine (pixels)

Control (n=18) vs. Diabetes (n=16)