THE EFFECTS OF TYPE 2 DIABETES ON CEREBROVASCULAR STRUCTURE AND FUNCTION

by

ALEX KEITH HARRIS

(Under the Direction of Adviye Ergul, M.D./Ph.D.)

ABSTRACT

Background: Cardiovascular disease, including stroke, is the leading cause of death in diabetes. Endothelin (ET-1) is upregulated in diabetes and previous studies have suggested that ET-1 may be involved in vascular remodeling and dysfunction in peripheral vessels. However, the effects of diabetes and ET-1 on the cerebrovasculature remained unknown. The central hypothesis of these studies was that: 1) cerebrovascular remodeling, characterized by ECM deposition and/or rearrangement, occurs in diabetes 2) ET-1 contributes to this process by altering the expression and activity of MMPs 3) diabetes induces vascular dysfunction in the form of either impaired relaxation or exacerbated contractile responses to vasoconstrictors. The rationale was that an understanding of the effects of diabetes on the cerebrovasculature would facilitate the design of future studies aimed at prevention of cerebrovascular disease in diabetes. Methods and Results: Control Wistar and diabetic Goto-Kakizaki (GK) rats, either untreated or subjected to 4 weeks of ET$_A$ or ET$_B$ receptor antagonism, were sacrificed at 18 weeks of age and vascular structure and function were assessed in middle cerebral arteries (MCA) and basilar arteries respectively. GKs exhibited remodeling of MCAs which was associated with elevated ET-1 levels and dysregulation of MMPs. In addition, basilar arteries from GKs were hyperreactive to ET-1 and had impaired endothelium dependent relaxation. ET$_A$ receptor antagonism restored MMP activity and decreased wall:lumen ratio in MCAs while decreasing ET-1 sensitivity and restoring ACh induced relaxation of basilar arteries. ET$_B$ receptor blockade significantly reduced medial thickening in MCAs and decreased basilar ET-1 sensitivity. Discussion: Vascular structural changes influence function by altering distensibility, elasticity, and compliance of blood vessels. In addition, disruption or modification of the extracellular matrix can uncover cryptic signals which in turn can begin a signal cascade leading to further cell proliferation and migration. These data suggest that Type-2 diabetes, devoid of confounding conditions such as hypertension and hyperlipidemia, induces structural as well as functional changes in the cerebrovasculature which are at least modulated, if not regulated by the endothelin system. In addition, the similarity of results obtained with ET$_A$/ ET$_B$ blockade indicate a greater involvement of ET$_B$ receptors in the development of diabetic vasculopathies.
INDEX WORDS: Diabetes, Endothelin, ET-1, Matrix Metalloproteinase, MMP, ET$_A$ receptor, ET$_B$ receptor, Goto-Kakizaki, Cerebrovascular Remodeling, Hyperglycemia, Basilar, MCA, Middle Cerebral Artery, ABT-627, A-192621
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DEDICATION

This dissertation is dedicated to my loving wife Melissa and my two beautiful boys, Ryan and Austin. Their love and support make it all worth while.
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CHAPTER 1

INTRODUCTION

Approximately 17 million Americans have diabetes mellitus and cardiovascular complications, including coronary artery disease, cerebrovascular disease and stroke, are the number one cause of death in this patient population. Although the risk of having a stroke is 3-fold higher in patients with diabetes, there has been substantially less research on diabetic complications involving the cerebrovasculature. Inasmuch, the molecular and cellular mechanisms that contribute to pathological changes in the structure of cerebral blood vessels that may predispose these patients to increased risk of stroke remain unclear. Endothelin-1 (ET-1), a potent vasoconstrictor produced predominantly by endothelial cells, promotes vascular smooth muscle cell (VSMC) growth and collagen deposition in the peripheral vasculature. However, to what extent ET-1 contributes to the pathological remodeling of the cerebrovasculature, and the mechanisms involved remain unknown. Matrix metalloproteinases (MMPs), a family of proteolytic enzymes, contribute significantly to vascular remodeling by degrading and reorganizing the extracellular matrix (ECM) as well as promoting VSMC growth and migration. The role of MMPs and the interaction between MMPs and ET-1 in cerebrovascular diabetic complications remain unclear. A better understanding of the regulation of vascular structure in diabetes would facilitate the development of novel targets to prevent cerebrovascular complications.
LITERATURE REVIEW

I. Vascular Disease in Diabetes

Diabetes mellitus causes abnormalities in many vascular beds (1). Diabetes produces a morphological thickening of the capillary basement membrane (2, 3) a decrease in the density of microvessels (4), endothelial cell degeneration (5), and an increase in platelet adhesion and aggregation (6). Research has shown that ischemic stroke incidence is increased in diabetics with fasting glucose levels >140 mg/dl (7). Also, the adjusted relative risk of atherosclerotic brain infarction more than doubles in men and women with diabetes compared to those non-diabetics (8).

The endothelium, which plays a major role in the regulation of vascular tonus, permeability, coagulation, and vascular smooth muscle cell growth, is an early target in diabetes and dysfunction of vascular endothelial cells participates in the diabetic vascular disease process (9, 10). For example, the release of vasodilator and antiproliferative mediators such as NO and prostaglandin-I\textsubscript{2} is decreased while production of ET-1 with vasoconstrictive and mitogenic properties is increased (10). These changes in endothelial function cause early hemodynamic abnormalities including impaired endothelium-dependent relaxation and ultimately vasoconstriction (11). Long term changes in blood flow and ischemia induce remodeling of the vessel and further contribute to diabetic vascular disease.

Studies have demonstrated that a strong correlation exists between diabetes and cerebrovascular disorders such as cerebral ischemia and stroke (12, 13). Diabetes not only increases the risk of stroke, but also contributes to increased stroke mortality and impaired recovery after stroke (14-16). Previous studies have reported increased
myogenic tone in experimental diabetes (17-19) as well as diminished endothelium derived relaxation in cerebral arteries from diabetic animals (1, 17, 20). However, the mechanisms by which diabetes enhances cerebrovascular disease is poorly understood.

II. Endothelin-1

Previous studies suggest that elevated plasma ET-1 levels may be involved in the pathogenesis of diabetic vasculopathies. Plasma ET-1 levels are elevated in both clinical and experimental diabetes (21-25). Basal ET-1 production in mesenteric arteries isolated from streptozotocin (STZ) induced diabetic rats is significantly higher than those obtained from non-diabetic rats (26). In diabetic animals, ET-1 receptor antagonism prevented ECM deposition in the retina as well as renal arteries (27, 28). Recently, Amiri et al demonstrated that endothelium restricted overexpression of ET-1 causes marked hypertrophic remodeling and endothelial dysfunction in mice (29).

In addition to its proliferative effects, growing evidence suggests that ET-1 also contributes to the development of vascular dysfunction in diabetes (30). Enhanced contractile responses to ET-1 have been demonstrated in aorta and peripheral vessels in diabetic animals (31-33). McIntyre et al have also shown that subcutaneous resistance arteries from Type-1 diabetic patients exhibit increased sensitivity to ET-1 (34). There are multiple factors involved in the precipitation of vascular remodeling and dysfunction in diabetes and ET-1 remains a likely candidate with the ability to mediate both functional and structural changes.
III. ET Receptors

The vasoactive and proliferative actions of ET-1 are mediated through two G-protein coupled receptors: ET\textsubscript{A} and ET\textsubscript{B} (Figure 1.1). ET\textsubscript{A} receptors are located on smooth muscle cells and mediate the contractile and mitogenic actions of ET. While functional studies have suggested that two ET\textsubscript{B} subtypes may exist (35-37) others have shown that genetic deletion of the ET\textsubscript{B} receptor abolishes the heterogeneous responses of the ET\textsubscript{B} receptor, suggesting only one gene (38). Nevertheless, a dual presence of the ET\textsubscript{B} receptor does exist within the vasculature. Hence, ET\textsubscript{B1} receptors, located on endothelial cells, mediate relaxation via cyclic GMP while ET\textsubscript{B2} receptors are located on smooth muscle cells (SMC) and behave as ET\textsubscript{A}s. These receptors may be differentially activated/regulated in varying pathological states. Studies in experimental diabetes have demonstrated attenuation of ET-1 hyperreactivity in cerebral arteries following ET\textsubscript{B} receptor blockade (32, 39, 40) producing effects similar to those observed with ET\textsubscript{A} antagonism. Others have observed increased levels of ET\textsubscript{B} receptor density and/or gene expression in different tissues from diabetic animals (41-43). While in these studies, ET\textsubscript{B} receptors appear to mediate potentially deleterious actions, others have
demonstrated potential vasculoprotective effects of these receptors. Murakoshi et al demonstrated that inhibition of the ET\textsubscript{B} receptor system, both through ET\textsubscript{B} receptor knockouts and pharmacological inhibition, resulted in enhanced neointimal hyperplasia and medial thickening in a vascular injury model (Figure 1.2) (44). Additionally, ET\textsubscript{B} receptor deficient animals were shown to be predisposed to pulmonary edema formation due to increased production of vascular endothelial growth factor (45). While Chuquet et al demonstrated that blockade of ET\textsubscript{B} receptors with the selective antagonist BQ-788 significantly increased infarct volume and decreased cerebral blood flow in an experimental stroke model (46), Stenman reported upregulation of contractile ET\textsubscript{B2} receptors following focal cerebral ischemia (47). This provides an alternate explanation in which ET\textsubscript{B} receptor blockade is potentially not impairing vascular protection, but rather it is decreasing myogenic tone and impairing autoregulation which contributes to decreased blood flow and exacerbated infarct. The role of ET receptors may be as variable as the tissue/disease state in which they exist. Therefore, an understating of the effects of these receptors in Type-2 diabetic cerebrovasculature is necessary for future development and use of antagonists.
IV. Vascular Remodeling and MMPs

Structural changes in blood vessels in vascular disease caused by hypertension and diabetes are characterized by a thickening of the vascular media and narrowing of the lumen diameter, which result in an increased media/lumen ratio. Also noted are abnormalities in the expression and/or localization of extracellular matrix (ECM) proteins such as collagen and laminin (48). The ECM is a dynamic structure and requires constant remodeling by synthesis of new component proteins and degradation of old components by MMPs, which are tightly regulated by tissue inhibitor of metalloproteinases (TIMP). The major source of ECM proteins in the vessel wall is vascular smooth muscle cells, which also synthesize MMPs and TIMPs.

In the vasculature, the MMP family includes MMP-1, MMP-2 and MMP-9. Interestingly, MMPs also promote VSMC growth and migration through activation of growth factors by proteolytic processing (49, 50). More importantly, MMPs activate membrane-bound proteins with growth-promoting properties via proteolytic cleavage (51-53). For example, cleavage of heparin binding epidermal growth factor (Hb-EGF) by an MMP dependent mechanism leads to activation of the EGF receptor promoting increased collagen synthesis (54). Both angiotensin-II and ET-1 enhance this transactivation process (52, 54, 55). However, the manner and circumstances in which the ET system affects MMPs
is not well elucidated. We have previously demonstrated that ET$_A$ receptor blockade attenuates increases in media to lumen ratio as well as vascular MMP dysregulation in diabetes (21). A recent study illustrated that increased aortic MMP-2 activity in spontaneously hypertensive rats is attenuated by non-selective ET antagonism with Bosentan (56). Podesser et al showed that treatment with the ET$_A$ selective antagonist, Sitaxsentan, reduces post MI left ventricular dilation as well as MMP activity in cardiac tissue (57). Clearly, endothelin modulates the expression and activity of MMPs which contribute to vascular remodeling. However, the exact relationship between ET-1, ET receptors, and the MMP system is not well defined. Elucidation of this system is necessary for a complete understanding of its effects on vascular structure and function in diabetes.
DISSE ETATION OBJECTIVES

The objective of the studies presented in this dissertation was to evaluate the effects of Type-2 diabetes on the structure and function of cerebral blood vessels and to determine the relative influence of endothelin-1 (ET-1) on these processes. It is well established that activation of the ET system occurs in both human and experimental diabetes. Previous studies have demonstrated that alterations of the endothelin system can influence vascular remodeling and dysfunction. However, the effects of hyperglycemia and/or ET-1 on the cerebrovasculature have remained unknown. The studies contained herein were designed to elucidate how experimental diabetes affects 1) blood vessel structure and function, and, 2) the regulation of proteins that govern matrix dynamics. Additionally, these studies also determined the effects of ET receptor antagonism on these vessels.

The central hypothesis of these studies was that cerebrovascular remodeling characterized by ECM deposition and/or rearrangement occurs in diabetes and that ET-1 contributes to this process by altering the expression and activity of MMPs. Additionally, it was hypothesized that diabetes will induce vascular dysfunction in the form of either decreased relaxation to vasodilators or increased contractile response to vasoconstrictors in isolated cerebral artery preparations. The rationale for these studies was that an understanding of the effects of diabetes on the cerebrovasculature would facilitate the design of future studies aimed at prevention of cerebrovascular disease in diabetes.
To test these hypotheses, three specific aims were proposed:

1. **Specific Aim 1:** Determine indices and markers of vascular remodeling including MMP expression/activity and vessel morphology in middle cerebral artery preparations from diabetic Goto-Kakizaki rats. Additionally, the effect of diabetes on cerebrovascular function will be assessed.
   
   - MMP expression and activity will be assessed, in MCAs from 18 week old diabetic and control rats, by immunoblotting and gelatin zymography.
   
   - Vascular structural changes will be determined in fixed cross sections of MCAs. These sections will be stained with Masson’s Trichrome and morphological measurements will be made to determine media to lumen ratios.
   
   - The effect of diabetes on vascular function will be assessed using basilar arteries from control and diabetic rats using the wire myograph method. ET-1 dose response curves (0.1-100 nm) will be constructed to determine the effect of diabetes on ET mediated vasoconstriction. Endothelium dependent relaxation will be assessed in serotonin (5-HT) pre-constricted vessels using acetylcholine (ACh, 1nm - 1µm).

2. **Specific Aim 2:** Determine the contribution of ET<sub>A</sub> receptors to cerebrovascular remodeling and dysfunction in experimental diabetes using ET<sub>A</sub> receptor antagonism.
   
   - Animals will be placed on chronic ET antagonism to determine the influence of the ET system, specifically the ET<sub>A</sub> receptor, on the cerebrovasculature in diabetes. Both control and diabetic animals will be divided into vehicle and
drug treated groups. Treated animals will receive the $\mathrm{ET}_A$ selective antagonist ABT-627 (5 mg/kg/d) orally in their drinking water beginning at 14 weeks of age and continuing for 4 weeks. During this time, blood pressure as well as blood glucose and weight will be monitored. Animals will be sacrificed at 18 weeks of age and vascular structure and function will be assessed as in Specific Aim 1.

3. **Specific Aim 3:** Determine the relative role of $\mathrm{ET}_B$ receptors in vascular remodeling and dysfunction in experimental diabetes.

Pharmacological inhibition using the $\mathrm{ET}_B$ selective antagonist A-192621 will be used to determine the contribution of $\mathrm{ET}_B$ receptors to vascular remodeling and dysfunction in diabetes. As in previous studies, animals will be divided into vehicle and treatment groups at 14 weeks of age. Drug treated animals will be given A-192621 (15 or 30 mg/kg/d divided into two doses) by oral gavage. Treatment will continue for 4 weeks and animals will be sacrificed at 18 weeks of age. As in the previous aims, basilar and middle cerebral arteries will be harvested for function and structure studies respectively.
REFERENCES


CHAPTER 2

TYPE-2 DIABETES CAUSES REMODELING OF CEREBROVASCULATURE VIA DIFFERENTIAL REGULATION OF MATRIX METalloPROTEINASES AND COLLAGEN SYNTHESIS: ROLE OF ENDOTHELIN-1

ABSTRACT

The risk of cerebrovascular disease is 4-6 fold higher in patients with diabetes. Vascular remodeling, characterized by extracellular matrix deposition and increased media-to-lumen ratio, occurs in diabetes and contributes to the development of complications. However, diabetes-induced changes in the cerebrovascular structure remain unknown. Endothelin-1 (ET-1), a potent vasoconstrictor with profibrotic properties, is chronically elevated in diabetes. In order to determine diabetes-mediated changes in the cerebrovasculature and the role ET-1 in this process, Type-2 diabetic Goto-Kakizaki (GK) rats were administered an ET_A receptor antagonist for 4 weeks. Middle cerebral arteries were harvested and studies were performed to determine vascular structure. Tissue and plasma ET-1 levels were increased in GKS compared to controls. Significant medial hypertrophy and collagen deposition resulted in increased wall-to-lumen ratio in diabetic rats that was reduced by ET_A receptor antagonism. Vascular MMP-2 activity was higher but MMP-1 levels were significantly reduced in GKS and MMP levels were restored to control levels by ET_A receptor antagonism. We conclude that ET-1 promotes cerebrovascular remodeling in Type-2 diabetes through differential regulation of MMPs. Augmented cerebrovascular remodeling may contribute to an increased risk of stroke in diabetes and ET_A receptor antagonism may offer a novel therapeutic target.
INTRODUCTION

Type-2 diabetes, a disease that affects more than 17 million Americans, holds a 2-6 fold increased risk for cerebrovascular disease and stroke (1; 2). Seventy percent of patients with recent stroke have overt diabetes or prediabetes characterized by impaired fasting glucose or impaired glucose tolerance (3). However, the underlying basis of this predisposition remains unclear. Diabetic vascular complications are associated with remodeling of the vessel wall in the retinal, renal and mesenteric circulations. However, diabetes-induced structural changes in the cerebral microvessels are unknown.

The endothelium is an early target in diabetes and dysfunction of vascular endothelial cells has a role in the diabetic vascular disease process (4). For example, the release of vasodilator and antiproliferative mediators such as NO and prostaglandin-$I_2$ is decreased while production of endothelin-1 (ET-1) is increased (5). A significant correlation has been observed between plasma ET-1 levels and diabetic complications. In addition to being vasoconstrictive, ET-1 is also mitogenic. In streptozotocin diabetes, non-selective ET receptor antagonism prevents extracellular matrix (ECM) deposition in the retina as well as in the mesenteric arteries providing evidence for a causal relationship (6; 7). Nonselective blockade of ET receptors also prevents increased myogenic tone of cerebral vessels in diabetes (8) but the effect on vascular structure and the underlying mechanisms remain to be identified.

The matrix metalloproteinases (MMPs) are a family of zinc dependent proteases that are responsible for extracellular matrix turnover (9). In pathological states such as cardiovascular disease, the MMPs may become deleterious due to dysregulation and
can result in tissue injury and inflammation. In experimental hypertension and atherosclerosis models for example, increased MMP activity contributes to cardiac and vascular complications (10-13). While it has been shown that diabetes enhances aortic MMP activity, mesangial cells grown under high glucose conditions have been shown to exhibit decreased MMP activity, which contributes to diabetic nephropathy via matrix accumulation. Clearly, the regulation of MMPs and their relative contribution to pathological processes varies between tissues, however the regulation of MMP expression and activity in the cerebrovasculature and especially in diabetes is not known. In order to identify diabetes-induced pathological changes in the cerebrovasculature that may contribute to the development of cerebrovascular complications in diabetes, the current study had three objectives: 1) assess the diabetes-induced structural changes in cerebral vessels, 2) determine the expression and activity of the MMP system in these vessels, and 3) determine to what extent ET-1 regulates cerebrovascular MMPs and remodeling. The central hypothesis was that increased MMP activity would be associated with hypertrophic remodeling of the cerebral vessels in the diabetic state and that blockade of the ET system would ameliorate this process.

RESEARCH DESIGN AND METHODS

Animal and Tissue Preparation

The Medical College of Georgia Institutional Animal Care and Use Committee (IACUC) approved all protocols. Male Wistar and Goto-Kakizaki (GK) rats were obtained from Taconic, Inc. (Germantown, NY, USA) at 8 weeks of age. All animals
were individually housed at the Medical College of Georgia’s animal care facility, were allowed access to food and water ad libitum, and were maintained on a 12/12 hour light/dark cycle. During housing, drinking water measurements, weight, and blood glucose measurements were performed twice weekly. Glucose measurements were taken from the tail vein and measured on a commercially available glucose meter (AccuChek, Roche Diagnostics, Indianapolis, IN). At 12 weeks of age, when all GK animals became overtly diabetic, telemetry transmitters for blood pressure measurements were implanted as previously reported (14). After a 2 week-recovery period, control and diabetic animals were administered the ET$_A$ selective antagonist, ABT-627 (5 mg/kg/day), or vehicle only (15;16). The drug was dissolved in drinking water at a concentration based on the animal’s weight and daily water consumption. Treatment was maintained until the time of sacrifice at 18 weeks of age. Animals were anesthetized with sodium pentobarbital and exsanguinated via the abdominal aorta. Upon sacrifice, the brain was removed and placed in ice-cold physiological saline solution. For immunohistochemistry, middle cerebral arteries (MCA) were perfused with Histogel (Richard Allen Scientific, Kalamazoo, MI), then excised and embedded in the same matrix. Upon gelling of the matrix, the embedded vessel was placed in 10% formalin for storage. For protein studies, vessels were excised, snap frozen in liquid nitrogen, and stored at -80°C.

**Plasma Measurements**

Plasma ET-1 and insulin were measured by specific ELISA kits from ALPCO Diagnostics (Windham, NH). Plasma triglycerides and cholesterol were measured using commercially available kits (Wako USA, Richmond, VA).
**Glucose Clearance Experiments**

Additional diabetic and control animals were restrained awake and baseline glucose was checked via the tail vein. A glucose bolus (0.6 mg/kg) was injected via the dorsal tail vein and glucose readings were taken at five-minute intervals for a total duration of one hour.

**Tissue Homogenization and Zymography**

Snap frozen MCAs were placed in modified RIPA buffer (50 mM Tris-HCl, 1 % Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1mM PMSF, 1 µg/ml aprotinin, leupeptin, pepstatin, 1 mM sodium orthovanadate, and 1 mM sodium fluoride) and sonicated at room temperature for 8-10 ten second bursts. Samples were placed on ice between sonications. Total protein was measured using the Bradford method (BioRad, Richmond, CA) in a 96-well format and read on a Quant spectrophotometer. Bovine serum albumin was used as a standard. On the day of the experiment, samples (20 µg protein/sample) were loaded onto 10% gelatin zymogram gels (BioRad, Richmond, CA) and separated by molecular weight under nonreducing conditions. The gels were then rinsed twice in 2.5% Triton X-100 and incubated for 24 hours in substrate buffer containing 21 mM Tris·HCl, 10 mM CaCl₂, 0.04% NaN₃. Gels were then stained by Coomassie blue R-250 followed by destaining in 55% methanol and 7% acetic acid. Lytic activity was viewed as clear bands on a dark blue background and was quantitated by densitometric analysis (Gel-Pro v 3.1, Media Cybernetics, Carlsbad, CA). Kaleidoscope protein standards (Bio-Rad, Richmond, CA) and recombinant MMP-2 and MMP-9 proteins (Calbiochem, San Diego, CA) were run in parallel with all samples. TIMP-2 levels were measured by ELISA (Amersham Biosciences, Piscataway, NJ).
Immunohistochemistry and Vessel Morphometry

MCA segments were fixed in 10% formalin, embedded in paraffin, sectioned at 4 microns, and mounted on treated slides. Slides were then deparaffinized, blocked (Super Block, Biogenex Labs, Inc., San Ramon, CA), and placed in PBS for 5 minutes. Slides were then incubated with ET-1 or CD68 primary antibody obtained from Peninsula Laboratories (San Carlos, CA), and Dako (Carpinteria, CA), respectively, at room temperature, washed, then incubated in secondary antibody (LSAB2-HRP Kit, Dako, Carpinteria, CA) followed by incubation with Streptavidin-HRP. Bound antibody was detected with DAB substrate kit. Additional slides were incubated with only the secondary antibody to determine non-specific staining. Slides were viewed using a Zeiss Axiovert microscope (Carl Zeiss, Inc., Thornwood, NY) and captured using Spot software. For morphometric studies, 4 µM vessel segments were subjected to Verhoeff van Gieson’s elastic staining or polychrome staining. Images were captured and analyzed using Spot software and wall to lumen ratios were calculated.

Western Blotting

Protein levels of MMPs (MMP-1 and MMP-2) were determined by immunoblotting as previously described (15). All blots were restained with anti-actin antibody for equal protein loading.

Statistical analysis

The profile of blood glucose changes over time was analyzed for group differences (control vs. diabetic) using a repeated measures analysis of variance where the group by time interaction was the test of interest. A Tukey adjustment was used for the post-hoc comparison of the groups at each time point. A rank transformation (17)
was applied to all other data prior to analysis to address issues of non-normality. A 2x2 analysis of variance was used to investigate the main effects of Disease (control vs. diabetic) and Drug (vehicle vs. ABT-627) and the interaction between Disease and Drug. Effects were considered statistically significant at p<0.05. SAS® version 8.2 was used for all analyses. Results are expressed as mean ± SEM.

RESULTS

Animal Data

Metabolic parameters for control and diabetic (GK) animals are summarized in Table 2.1. Diabetic animals were significantly smaller than control and ETₐ antagonism did not affect animal weight. GK animals displayed mildly elevated blood glucose and blood pressure without hyperlipidemia and hyperinsulinemia. Experiments were performed in diabetic and control animals to assess glucose clearance capacity. Figure 2.1 indicates that the maximal response to glucose challenge was significantly higher in untreated diabetic animals versus control (259% of baseline vs. 168%) (p<0.05). Additionally, after 30 minutes, diabetic animals exhibited a decreased ability to clear glucose and return to normal levels providing evidence for impaired glucose tolerance (p<0.05).

Local and Systemic ET-1 Levels

Plasma ET-1 levels were elevated approximately three-fold in diabetic animals (p=0.001) (Figure 2.2A). Tissue ET-1 was assessed by immunohistochemistry and as shown in Figure 2.2B, there was enhanced endothelial and adventitial staining in the MCAs from diabetic animals as compared to controls. Staining in the absence of the
primary ET-1 antibody was determined to eliminate nonspecific staining by the secondary antibody.

Cerebrovascular MMP Expression and Activity

MMP activity in MCAs from treated and untreated diabetic GK and control Wistar rats was assessed using gelatin zymography. A representative zymogram is shown in Figure 2.3A. Lytic activity, corresponding with the molecular weight of active MMP-2, was detected in all samples. Densitometric analysis demonstrated that MMP-2 activity was increased in diabetes (p<0.0001) and that ET\textsubscript{A} receptor antagonism prevented this increase in activation (p=0.001 vs. untreated GK) (Figure 2.3A). Since TIMP-2 is the endogenous inhibitor of MMP-2, TIMP-2 levels were measured to determine whether the increase in MMP-2 activity arises from a decrease in its inhibitor. Although there was no significant difference between the control and diabetes groups, there was a trend for increased TIMP-2 levels with ET\textsubscript{A} antagonism.

In order to determine whether increased MMP-2 activity is due to an increase in protein levels, total MMP-2 protein in MCAs was assessed by immunoblotting. Two bands of 72 kD and 67 kD were detected corresponding to the zymogen and cleaved active forms respectively. A representative immunoblot is shown in Figure 2.4A. Densitometric analysis of both bands indicated a four-fold increase in MMP-2 protein in diabetes (p<0.003 vs. control). However, unlike the zymography studies, ET\textsubscript{A} receptor antagonism did not cause a change in MMP-2 protein levels (Figure 2.4A). Since MMP-1 is the major MMP that degrades fibrillar collagen, protein levels were determined in MCAs by immunoblotting. There was a significant decrease in MMP-1 levels in GKs
(p=0.034) and a trend toward restoration of MMP-1 levels to control values with ET\textsubscript{A} receptor antagonism (Figure 2.4B).

**Vascular Structure**

Verhoeff van Gieson’s (VVG) elastic staining was performed to determine vessel diameter and wall thickness (Figure 2.5A). There was a two-fold increase in wall:lumen ratio in GK rats that was significantly reduced by ET\textsubscript{A} receptor antagonism (Figure 2.5B) (p=0.042). Compared to control rats, there was increased staining for smooth muscle fibers and collagen as evidenced by deep purple staining by VVG in the cross sections from GK rats. These changes were detected mainly in the medial layer. To further evaluate the collagen deposition dynamics, picro polychrome staining was used to differentiate the old and newly laid collagen. In untreated GKS, there was increased blue staining for new collagen, masking the red staining for old collagen, indicating increased collagen synthesis. ET\textsubscript{A} antagonism reduced the staining pattern for new collagen. In order to determine whether and to what extent inflammation contributes to the increased wall thickness and collagen deposition, the same vascular cross sections were immunostained with CD68 antibody, a marker for macrophages. There was no difference between groups (data not shown).

**DISCUSSION**

It is well established that diabetes causes hypertrophic remodeling of the peripheral vasculature characterized by reduced lumen diameter, media enlargement, abnormalities of expression and/or localization of ECM components such as collagen and laminin deposition in the vessel wall and accelerated formation of atherosclerotic
plaques and intimal proliferation (7; 18-21). It is also known that Type-2 diabetes, a disease that affects more than 17 million Americans, holds a 2-6 fold increased risk for cerebrovascular disease and stroke. Furthermore, diabetes increases the risk of microvascular hemorrhage and poor outcome of stroke. The majority of experimental stroke models are induced by MCA occlusion and while it is known that the integrity of cerebral blood vessels is very critical in the pathophysiology of stroke, diabetes-induced changes in MCA structure have remained unknown. This study was designed to look at structural changes and potential mechanisms of altered matrix dynamics in the cerebral microvasculature in diabetes. Our findings demonstrate for the first time that mild hyperglycemia for 4-6 weeks stimulates the local production of ET-1 and causes medial thickening in the cerebrovasculature which are associated with increased gelatinase (MMP-2) activity and decreased collagenase (MMP-1) levels. Furthermore, MMP activation and increased wall:lumen ratio can be partially prevented by the administration of an ET$_A$ receptor antagonist providing evidence that ET-1 is in part responsible for pathological remodeling of the cerebral vessels in diabetes.

The chemically-induced STZ model of Type-1 diabetes, the most commonly used experimental model of diabetes, displays highly elevated glucose levels. There is still a need for spontaneous models of Type-2 diabetes in which blood glucose levels are comparable to those seen in patients. GK rats generally spontaneously manifest hyperglycemia by approximately 8-10 weeks of age (22). It has been shown that these animals retain greater than 40% of their total beta-cell mass (a number similar to human Type-2 diabetes) but have impaired glucose-induced insulin release (23-25). In addition, treatment of GK rats with nateglinide, an insulin secretagogue, reduces post-prandial
hyperglycemia and elicits early phase insulin secretion, a phenomenon not observed in Type-1 diabetes (26; 27). Insulin resistance and hyperlipidemia often accompany Type-2 diabetes and the presence of these risk factors in GK rats has been controversial (22; 28; 29). Thus, we specifically assessed the presence of these risk factors in our colony. GKs developed hyperglycemia by approximately 12 weeks of age. While plasma insulin levels are not elevated, impaired glucose tolerance tests demonstrate a defect in clearing glucose in GK rats when compared to normal Wistar rats, which serve as control for this model. Metabolic parameters demonstrate that by 18 weeks of age (6 weeks of diabetes) this model displays significant hyperglycemia and a slight elevation in blood pressure without hyperlipidemia and hyperinsulinemia. Recent studies identified that glucose intolerance is another risk factor for CVD. The Hoorn Study reported increased arterial stiffness in glucose intolerance and Type-2 diabetes (30). Inasmuch, the GK rat model serves as a good model to study the impact of moderate changes in blood glucose on cerebrovascular complications of diabetes. Our findings are significant in that even under a relatively short duration of mildly hyperglycemic conditions without the confounding effects of hyperlipidemia and insulin resistance, there are striking pathological changes in the cerebrovascular structure.

Plasma ET-1 levels are elevated in Type-1 and Type-2, as well as experimental diabetes (31-33). In Type-1 diabetes, mixed ET receptor blockade significantly reduced mesenteric wall-lumen-ratio and ECM deposition (7). Endothelial overexpression of human ET-1 has been recently shown to significantly increase media-to-lumen ratio in murine mesenteric arteries (34). Nonselective blockade of ET receptors also prevents increased myogenic tone of cerebral vessels in diabetes but the effect on vascular
structure remained to be identified. Several studies have linked ET-1 to the regulation of MMPs. ET_A receptor antagonism has been reported to decrease collagen accumulation and improve MMP-2 activity in kidneys from stroke-prone spontaneously hypertensive rats (35). We have recently shown that environmental stress upregulates vascular MMP-2 activity via stimulation of ET-1 synthesis and ET_A activation (15). Therefore, we investigated the regulation of cerebrovascular MMPs that can degrade collagen (MMP-1) and gelatin (MMP-2 and MMP-9). Increased ECM protein synthesis, diminished MMP activity and/or increased TIMP activity all could contribute to matrix accumulation, which is a late event in the vascular remodeling process (36). Interestingly, recent studies demonstrated that MMP activation also contributes to VSMC growth and migration, as well as increased collagen synthesis via several mechanisms. MMPs, especially MMP-9, degrade basement membrane and internal elastic lamina disrupting the boundaries between vascular layers and facilitating VSMC migration (37; 38). Additionally, the breakdown of fibrillar collagen unmask cryptic integrin signals buried in the ECM that serve as chemotactic stimuli for VSMC migration (38; 39). More importantly, MMPs activate membrane-bound proteins with growth-promoting properties via proteolytic cleavage (40-42). For example, cleavage of heparin binding epidermal growth factor (Hb-EGF) by an MMP dependent mechanism leads to activation of the EGF receptor promoting increased collagen synthesis (43). It has been demonstrated that both angiotensin-II and ET-1 enhance this transactivation process (41; 43; 44). In our study, we found ET-1 mediated increases in MMP-2 expression and activity. In addition, ET_A receptor antagonism attenuated new collagen deposition in diabetic animals. However, it has to be recognized that with the available data, we cannot differentiate whether the
improvement of wall:lumen ratio in the ABT-627 treated GK group is a direct effect of receptor blockade or due to the reduction of blood pressure in this group. It is conceivable that in the GK model, ET-1 promotes MMP-2 activation, which results in increased collagen synthesis. At the same time, ET-1 downregulates MMP-1 protein levels promoting collagen deposition. These data provide evidence that ET-1 promotes matrix accumulation via differential regulation of MMP enzymes involved in collagen dynamics.

Our results may have post-ischemic relevance in addition to the possible stroke potentiating effects of MMPs in diabetes. The blood brain barrier exists as a physical barrier to solute transport in the brain. During cerebral ischemia, the integrity of this barrier comes under attack leading to increased permeability of the cerebral vessels (45; 46). This breakdown allows for leakage of blood into the perivascular spaces resulting in further damage to the already ischemic tissue. Several studies have demonstrated increased MMP activity and matrix degradation following focal cerebral ischemia (46-50). Our findings of increased basal MMP activity in diabetes may account for poor stroke outcomes in hyperglycemic patients.

We conclude that even mild Type-2 diabetes, without the confounding effects of hyperinsulinemia and hyperlipidemia, causes significant cerebrovascular remodeling via the modulation of the MMPs that are regulated by ET-1. Altered MMP activity might contribute to increased risk of stroke in diabetes and ETA receptor blockade may provide an effective therapeutic intervention for reducing ischemic brain damage in diabetes.
Acknowledgements

This work was supported by grants from NIH (HL076236-01), American Heart Association Scientist Development Grant, American Diabetes Association Research grant and Pfizer Atorvastatin Research Award to Adviye Ergul and an AHA Southeast Affiliate Predoctoral Fellowship Award to Alex K. Harris. The authors wish to thank Abbott Laboratories for the ABT627 compound.
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*Decreased blood glucose excursion by nateglinide ameliorated neuropathic changes in Goto-Kakizaki rats, an animal model of non-obese type 2 diabetes.* Metabolism 51:1452-1457, 2002


<table>
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<tr>
<th></th>
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<th>Control + ABT-627</th>
<th>GK</th>
<th>GK + ABT-627</th>
</tr>
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<td>ET-1</td>
<td>0.3 ± 0.04</td>
<td>0.3 ± 0.07 (6)</td>
<td>0.8 ± 0.1*</td>
<td>0.5 ± 0.06*</td>
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<td>168 ± 21**</td>
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<tr>
<td>Insulin</td>
<td>2.3 ± 0.2</td>
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<td>1.5 ± 0.2**</td>
<td>0.7 ± 0.1**</td>
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<tr>
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<td>607 ± 11 (5)</td>
<td>411 ± 36**</td>
<td>409 ± 29**</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>121 ± 4</td>
<td>124 ± 7 (10)</td>
<td>139 ± 11 (5)</td>
<td>125 ± 5</td>
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<tr>
<td>Triglycerides</td>
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<td>25 ± 4**</td>
<td>14 ± 2**</td>
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<tr>
<td>Blood Pressure</td>
<td>104 ± 2</td>
<td>98 ± 5**** (3)</td>
<td>121 ± 1**</td>
<td>112 ± 1******</td>
</tr>
</tbody>
</table>

Mean ± SEM (n)

*p=0.001, GK vs Control
**p=0.0001, GK vs Control
***p=0.04, Vehicle vs ABT-627
****p=0.007, Vehicle vs ABT-627
**FIGURE LEGENDS**

**Figure 2.1.** Goto-Kakizaki diabetic rats have impaired glucose tolerance. Untreated control and diabetic animals were given IV glucose and blood glucose was monitored for 1 hour. Diabetic animals exhibited higher maximal responses as well as impaired ability to clear glucose. *p<0.05 vs. control

**Figure 2.2.** Plasma and tissue ET-1 levels are increased in diabetes. **A.** Circulating plasma ET-1 levels were measured by ELISA. **B.** Frozen MCA cross-sections were immunostained with an anti-ET-1 antibody (n=3/group). Nonspecific staining was determined in the absence of primary antibody. *p<0.001 vs. control

**Figure 2.3.** MMP-2 activity is increased in diabetes. **A.** Representative zymogram showing changes in vascular MMP-2 activity and densitometric analysis of lytic bands indicates an increase in MMP-2 activity that is ameliorated by the ET<sub>A</sub> receptor blockade. **B.** Tissue TIMP-2 levels were measured by ELISA. *p<0.0001 vs control, **p<0.001 vs GK.

**Figure 2.4.** MMP proteins are differentially regulated in diabetes. **A.** Representative immunoblot demonstrating increased MMP-2 expression in vascular homogenates and densitometric analysis of immunoreactive bands indicates that MMP-2 protein is increased in diabetic vascular disease. **B.** Representative immunoblots demonstrating decreased expression of MMP-1 in diabetes. *p=0.003 vs control, **p=0.034 vs control.
Figure 2.5. Vessel segments were analyzed for morphological changes and collagen deposition by VVG A. and picropolychrome B. staining. Diabetes induced a two-fold increase in wall to lumen ratio that was significantly reduced in the ABT-627 treated GK group. Qualitative assessment of differential collagen staining by picropolychrome staining indicates both new (red) and old (blue) collagen staining in the GKs. ET$_A$ antagonism reduces new collagen indicating improved collagen synthesis in the GKs. p=0.042.
FIGURE 2.1

Glucose Clearance

% Change from Baseline

Time (min)

- Control
- GK

* p<0.05
FIGURE 2.3

A. Optical Density (pixels)

- C: n=8
- C + ABT: n=5
- GK: n=9
- GK + ABT: n=5

B. TIMP-2 (ng/µg Protein)

- C: n=6
- C + ABT: n=5
- GK: n=3
- GK + ABT: n=5
FIGURE 2.4

A.  

MMP-2

B.  

MMP-1

OD (pixels)

C  C + ABT  GK  GK + ABT

n=6  n=5  n=3  n=5

n=6  n=5  n=3  n=5
FIGURE 2.5

A.

Diabetic  Control

-ABT

+ABT

B.

Diabetic  Control

-ABT

+ABT

C.

Wall: Lumen Ratio

![Graph showing Wall: Lumen Ratio comparisons for different conditions.]

- C: n=3
- C + ABT: n=3
- GK: n=4
- GK + ABT: n=3

* p < 0.05
** p < 0.01
CHAPTER 3

MICRO VS. MACROVASCULAR DYSFUNCTION IN TYPE-2 DIABETES:
DIFFERENCES IN CONTRACTILE RESPONSES TO ENDOTHELIN-1

\[1\] Sachidanandam, K., \(^1\)Harris, A.K., Hutchinson, J., Ergul, A. *Experimental Biology and Medicine, in press*. NOTE: First two authors contributed equally to this work. Reprinted with the permission of the Society for Experimental Biology and Medicine.
ABSTRACT

Vascular dysfunction characterized by a hyperreactivity to vasoconstrictors and/or impaired vascular relaxation contributes to increased incidence of cardiovascular disease in diabetes. Endothelin-1 (ET-1), a potent vasoconstrictor, is chronically elevated in diabetes. However, the role of ET-1 in resistance vs. larger vessel function in mild diabetes remains unknown. Accordingly, this study investigated vascular function of third order mesenteric arteries and basilar arteries in control Wistar and Goto-Kakizaki (GK) rats, a model of mild Type-2 diabetes. Six-weeks after the onset of diabetes, contractile responses to ET-1 (0.1-100 nM) and relaxation responses to acetylcholine (ACh, 1 nM-10 uM) in vessels preconstricted (baseline + 60%) with serotonin (5-HT) were assessed by myograph studies in the presence or absence of a NOS inhibitor (L-NNA). Maximum contractile response to ET-1 was augmented in mesenteric vessels (155 ± 18 in GK and 81 ± 6 % in control, n=5-7) but not in the basilar artery (134 ± 29 in GK vs 107 ± 17% in control, n=4/group). However, vascular relaxation was impaired in the basilar arteries (22 ± 4% in GK and 53 ± 7% in control, n=4/group) but not in mesenteric arteries of GK rats. Inhibition of NOS decreased the relaxation response of basilar arteries to 15 ± 8 and 42 ± 5% in GK and control rats, respectively, whereas in resistance vessels, corresponding values were 56 ± 7% and 89 ± 3% (versus 90 ± 2% and 112 ± 3% without NOS blockade) indicating the involvement of different vasorelaxation-promoting pathways in these vascular beds. These findings provide evidence that the ET system is activated even under mild hyperglycemia and contributes to the hyperreactivity of resistance vessels, which may play an important role in elevated blood pressure in Type-2 diabetes.
INTRODUCTION

Diabetes mellitus is a major health issue and, with nearly 75,000 deaths in the United States in 2002, is also a leading cause of mortality. Of those deaths, it is estimated that 65-75% can be attributed to cardiovascular disease (CVD) including hypertension and stroke (1). While it is known that diabetes is a major risk factor for CVD (2, 3), the mechanisms that potentiate this risk are not fully understood.

Vascular dysfunction, characterized by impaired relaxation to vasodilators or exacerbated response to vasoconstrictors, co-exists in many disease states such as hypertension and diabetes. In general, these diseases alter vascular responses to vasoactive substances, leading to increased basal tone and an inability to respond normally to stimuli. Studies in streptozotocin (STZ)-induced Type-1 diabetes have demonstrated decreased endothelium mediated relaxation in both mesenteric and cerebral (basilar) arteries (4-6). Additionally, similar results have been obtained in superior mesenteric arteries from the Type-2 diabetic Goto-Kakizaki rat (7-9). However, the effect of Type-2 diabetes on mesenteric microvessels and cerebral vessels has remained unknown.

The potent vasoconstrictor ET-1 is chronically up-regulated in diabetes (10-12) and may contribute to cardiovascular disease and diabetic complications (13, 14). Studies have demonstrated enhanced contractile responses in the mesenteric and basilar arteries in STZ treated rats (15, 16) as well as in basilar arteries from alloxan induced diabetic rabbits (17). Furthermore, Dumont et.al. have shown that chronic endothelin receptor antagonism abolishes increased myogenic tone in diabetic cerebral arteries (18).
The endogenous vasodilator nitric oxide (NO) plays an important role in the regulation of vascular tonus in response to both molecular and physical signals to increase levels of cyclic GMP and dilation. Decreased NO bioavailability, either through decreased production, primarily by endothelial nitric oxide synthase, or increased scavenging by reactive oxygen species (ROS), has been shown to impair vascular relaxation (19-22). Thus, within the scope of diabetes, it is important to understand the relative dependence of the vasculature on nitric oxide.

The objective of the current study was to compare and contrast the effects of hyperglycemia on different vascular beds in a model of Type-2 diabetes. Specifically, maximal contractile responses and endothelium-dependent relaxation responses were investigated in third order mesenteric and basilar arteries from Type-2 diabetic Goto-Kakizaki rats. Additionally, we sought to determine the relative contribution of NO-mediated relaxation in these vessels.

MATERIALS AND METHODS

Animals

All experiments were performed on male Wistar (Harlan, Indianapolis, IN) and Goto-Kakizaki (in-house bred, derived from the Tampa colony) rats (23, 24). The animals were housed at the Medical College of Georgia 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 until sacrifice at 18 weeks of age.
**Surgical procedures**

Animals were anesthesized and decapitated. The brain was quickly excised for isolation of basilar arteries. The mesenteric bed was then harvested and third order mesenteric arteries were isolated for vascular function studies.

**Determination of vascular function**

Isometric tension exerted by the vessels was recorded via a force transducer using the wire-myograph technique (Danish Myo Technologies, Denmark). The myograph chambers were filled with Kreb’s buffer (NaCl 118.3, NaHCO₃ 25, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 1.5 and Dextrose 11.1 mM), gassed with 95% O₂ and 5% CO₂ and maintained at 37°C. Vessel segments were mounted in the chamber using 40 µm-thin wires and adjusted to a baseline tension (~0.5g for basilar and 1g for mesentery). After stabilization, the vessels were challenged with 70 mM KCl and only those vessels that had 70% response above the baseline were considered viable. Cumulative dose response curves to ET-1 (0.1-100 nM) were generated and the force generated was expressed as % change of baseline. Endothelium-dependent relaxation to acetylcholine (ACh, 1 nM-1 µM) was assessed after vessels were constricted to 60% of the baseline tension with serotonin (5-HT) either alone or with a 30 min pre-incubation with the e-NOS inhibitor, L-NNA (100 nM). Sensitivity (EC₅₀) and maximum response (R_max) values were calculated from the respective dose-response equations.

**Statistical analysis**

EC₅₀ and R_max within-group differences were determined by t-tests. For multiple group comparisons within and between vascular beds, a one-way or two-way analysis of variance (ANOVA) was done accordingly, with a post hoc Bonferroni test. A repeated
measures ANOVA was used to determine group differences (Diabetic vs. Control) across the ET-1 concentrations. Post-hoc group comparisons at each concentration used a Tukey's adjustment for the multiple comparisons. An area under the curve (AUC) was calculated for each rat and comparisons between groups for AUC differences were determined using the exact Wilcoxon rank sum test. Statistical significance was determined at p<0.05. NCSS 2004 was used for the AUC determination and SAS 9.1.3 was used for all other analyses.

RESULTS

Animal data

Metabolic parameters for control and diabetic (GK) animals are summarized in Table 3.1. Diabetic animals were significantly smaller than control animals and displayed mildly elevated blood glucose and a small but not significant increase in blood pressure.

ET-1 dose-response

Mesenteric arteries of GK rats were hyperreactive to ET-1 (R_{max} 155 ± 18%) compared to the control rats (81 ± 6%), though their sensitivity was not significantly altered (Figure 3.1A, Table 3.2). There was a significant interaction between groups across ET-1 concentration range (p<0.0001) indicating a differential effect on responses to ET-1 between diabetic and control rats. The response was significantly different (all p<0.0001) for concentrations from 1-100 nM. The mean ± SD AUC for the control rats was 155.6 ± 46.7 and was 406.3 ± 103.1 for the diabetic rats. There was a significant different between the groups (p=0.0025). However, in the basilar arteries, sensitivity to
ET-1 was greatly augmented (4.2 ± 2.7 vs 13.1 ± 2.0 nM) whereas the maximum constriction was not significantly different (134 ± 29 vs 107 ± 17%) (Figure 3.2A, Table 3.2).

**Endothelium-dependent relaxation**

As shown in Figure 3.1B, there was no impaired relaxation to Ach in the mesenteric arteries of GKS just as in the controls. In the presence of L-NNA, there was a greater decrease in relaxation in the GKS (56 ± 7%) compared to the control animals (89 ± 3%) suggesting a greater involvement of NO-mediated relaxation in the diabetic group. While the EC\textsubscript{50} values increased in both groups indicating decreased sensitivity in the presence of L-NNA, there was no significant difference between controls and GKS. In the cerebral circulation, endothelium-dependent relaxation was impaired to a greater degree (Figure 3.2B, Table 3.2). The basilar arteries of GKS relax only 22 ± 4%, whereas the control rat arteries relax 53 ± 7% after preconstriction with 5-HT. Sensitivity to ACh is also greatly reduced in the GK rats in comparison to the control rats (20.4 ± 2 and 63 ± 2 nM) (Figure 3.2B, Table 3.2). In the presence of L-NNA, impaired relaxation of the basilar arteries is augmented even further in the GKS compared to controls.

**Vascular responses in mesenteric arteries versus basilar arteries**

Vascular constriction and relaxation responses were greatly altered in the two vascular beds in the GK rats. In the mesenteric arteries, the sensitivity to ET-1 was augmented nearly 4-fold in comparison to the basilar arteries (Figure 3.3A, Table 3.2), the magnitude of constriction however increasing comparably. In relaxation responses to ACh, there was no impairment of endothelium-dependent relaxation in the mesenteric
DISCUSSION

While it is well established that endothelial dysfunction underlies vascular complications of diabetes (19, 25), past studies focused mostly in streptozotocin-induced Type-1 diabetes or in Type-2 models associated with obesity such as the Zucker rat (26, 27). This study addressed the hypotheses that vascular responses to the potent constrictor ET-1 and the endothelium-dependent dilator Ach in small resistance vessels versus larger vessels differ in a non-obese model of Type-2 diabetes that displays mild hyperglycemia. Using the GK rat, we demonstrated that mesenteric resistance arteries are hyperreactive to ET-1 whereas basilar arteries are more sensitive to the same stimulus. We also provided evidence that after six weeks of mild diabetes, endothelium-dependent relaxation is not impaired in the mesenteric bed but is decreased about 80% in the cerebral vessels.

The endothelium plays an important role in the control of vascular tone, in part through the release of vasoactive factors, and in diabetes the imbalance of vasodilators and vasoconstrictors contributes to the increased risk of CVD including hypertension and stroke in diabetes (28). Since resistance arteries play an important role in the regulation of blood pressure and relatively larger arteries such as the basilar artery participate in the regulation of cerebral vascular resistance that is critical in the pathophysiology of stroke, this study was designed to compare and contrast the vascular reactivity of these two beds in diabetic GK and control rats. Cheng et al
reported that the GK rats display endothelial dysfunction and impaired vasorelaxation of superior mesenteric arteries that is associated with salt-sensitive hypertension (7). Witte et al demonstrated increased blood pressure and decreased endothelium-dependent relaxation in the mesenteric bed (9). In the current study, we did not observe any impairment of the relaxation response to Ach and the data indicate a greater involvement of the NO pathway in the relaxation response in the GK rats compared to controls as evidenced by 50 vs 10 % blockade, respectively, in the presence of L-NNA. Another group also reported normal endothelium-dependent relaxation in this model (29). These differences may be due to the age of the animals used or the size of the mesenteric arteries. In the current study we used segments 200-250 µm in diameter, which allowed the insertion of two pieces of 40 µm wire for the myograph. Witte et al reported using 120 µm wire in their system indicating the use of a superior mesenteric artery segment.

Several studies reported impaired relaxation of the middle cerebral artery in insulin resistant rats prior to the development of overt diabetes, however vascular reactivity changes in the cerebral circulation in Type-2 diabetes are largely unknown. Recently, Matsumoto et al reported that contraction induced by blockade of NOS is significantly less in the basilar artery of STZ-diabetic rats, suggesting that the NO pathway is already dysfunctional in the cerebral circulation (16). The current study also demonstrated that endothelium-dependent relaxation is severely impaired in basilar arteries. In the presence of L-NNA, basilar arteries of GKs fail to relax, whereas there is approximately 50% relaxation in the mesenteric circulation, providing evidence that the
NOS pathway is dysfunctional in the cerebral circulation of Type-2 diabetic animals as well and further blockade with L-NNA does not have an impact on maximal relaxation.

It is well-established that circulating and local ET-1 levels are elevated in both clinical and experimental Type-2 diabetes (11, 30-32). Antagonism of ET receptors prevents diabetic complications (33-35). In the current study we found that the ET-1 response is enhanced in the mesenteric bed. Katakam et al reported an augmented ET-1 response and ET receptor expression in insulin-resistant rats providing support for an activated ET system in diabetes (36). The current data demonstrate that basilar arteries are more sensitive to ET-1. Matsumoto and colleagues reported enhanced contractile response in Type-1 diabetes, again providing further support for the findings of the current study (16).

In conclusion, this study demonstrates differences in macro vs microvascular dysfunction in a mild model of Type-2 diabetes. The lack of impaired function of the mesenteric microvessels in contrast to a decreased relaxation response in the basilar arteries of GKS could possibly suggest that in the cerebral circulation, there is a greater degree of impaired relaxation early on in diabetes than in the gut. Hyperreactivity to ET-1 however is augmented in the mesenteric and not in the basilar arteries, suggesting that different pathways could be involved in different vascular beds, each contributing independently to vascular dysfunction.
Acknowledgements

This work was supported by grants from NIH (HL076236-01), American Diabetes Association Research Award and Philip Morris Research Award to Adviye Ergul.
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**TABLE 3.1**

*Table 3.1. Metabolic parameters of Control Wistar and GK rats.*

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<thead>
<tr>
<th>Metabolic Parameters</th>
<th>Control</th>
<th>GK</th>
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<td>Body weight (g)</td>
<td>502 ± 11</td>
<td>359 ± 7*</td>
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<td>Blood glucose (mg/dl)</td>
<td>116 ± 5</td>
<td>239 ± 23*</td>
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<tr>
<td>Mean Arterial Pressure (MAP, mm Hg)</td>
<td>101 ± 2</td>
<td>110 ± 7</td>
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*p<0.05 vs control*
Table 3.2. Sensitivity (EC$_{50}$) and magnitude ($R_{\text{max}}$) of vascular responses to ET-1 and ACh in the absence or presence of e-NOS inhibitor L-NNA (100 nM) in mesenteric and basilar arteries of control Wistar and Goto-Kakizaki (GK) rats.

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<td>GK</td>
<td>Control</td>
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<tr>
<td>ET-1</td>
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<td>L-NNA/ACh</td>
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<td>R$_{\text{max}}$ (%)</td>
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<td>ET-1</td>
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<td>L-NNA/ACh</td>
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*p<0.001 vs Control, n = 4-9/group

** p<0.05 vs GK Basilar, n = 4-9/group
FIGURE LEGENDS

**Figure 3.1.** A. Constriction responses to ET-1 in mesenteric arteries of control (Wistar) and diabetic (GK) rats: The magnitude of constriction (Rmax) was increased in GK’s, although sensitivity (EC$_{50}$) to ET-1 was not altered. B. Relaxation responses to ACh in the absence or presence of e-NOS inhibitor L-NNA (100 nM) in mesenteric arteries of control and GK rats following pre-constriction with 5-HT. *R$_{max}$ p<0.0001 vs control, **R$_{max}$ p<0.05 vs control LNNA+ACh.

**Figure 3.2.** A. Constriction responses to ET-1 in basilar arteries of control and GK rats. B. Relaxation to ACh in the absence or presence of e-NOS inhibitor L-NNA (100 nM) in basilar arteries following pre-constriction with 5-HT. *R$_{max}$ p<0.05 vs control, **R$_{max}$ p<0.05 vs GK, &EC$_{50}$ p<0.05 vs control, &&EC$_{50}$ p<0.05 vs GK.

**Figure 3.3.** A. Comparison of constriction responses to ET-1 in mesenteric and basilar arteries of GK rats: GK rats were more sensitive to ET-1, the magnitude of constriction however, unaffected. B. Relaxation responses to ACh in absence or presence of e-NOS inhibitor L-NNA demonstrated that relaxation to ACh was impaired in the basilar arteries, but not in the mesenteric arteries. With NO blockade, about 50% relaxation is abolished in the mesentery, but relaxation in the basilar artery is almost null. #EC$_{50}$ p<0.05 vs mesentery. * R$_{max}$ p<0.05 vs mesentery ACh, &EC$_{50}$ p<0.05 vs mesentery ACh
FIGURE 3.2

A.

% Constriction from baseline

ET-1 concentration log[M]

Control
Diabetic

B.

Relaxation to ACh (% of 5-HT preconstriction)

ACh concentration log[M]

Control ACh
Control LNNA+ACh
Diabetic ACh
Diabetic LNNA+ACh

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CHAPTER 4
EFFECT OF CHRONIC ENDOTHELIN ANTAGONISM ON TYPE-2 DIABETIC CEREBROVASCULAR FUNCTION

Harris, A.K., Sachidanandam, K., Hutchinson, J., Ergul, A. To be submitted to The Journal of Cardiovascular Pharmacology.
ABSTRACT

Changes in both vascular function and structure may contribute to increased stroke risk as well as worsened stroke outcome in diabetes. Conduit arteries such as basilar and middle cerebral artery (MCA) contribute significantly to microvascular pressure and we have previously shown that Type-2 diabetes causes remodeling of MCAs. Since previous studies have demonstrated enhanced ET-1 induced vasoconstriction and impaired endothelium mediated relaxation of basilar arteries in streptozotocin induced diabetes, the current study sought to elucidate the potential vascular roles of the ET receptors in a non-obese, normotensive model of Type-2 diabetes, the Goto-Kakizaki (GK) rat.

Fourteen week old GK or control Wistar rats were treated with antagonists to either ET$_A$ (ABT-627, 5 mg/kg/d) or ET$_B$ (A-192621, 15 or 30 mg/kg/d) receptors for four weeks. Basilar arteries were harvested and vascular function assessed using a wire myograph. Compared to control, GK basilar arteries were hypersensitive to ET-1 (EC$_{50}$, 3.8 ± 1.6 vs 13 ± 2, p<0.05). Sensitivity in the GK basilar arteries was significantly attenuated by ET$_A$ receptor antagonism (EC$_{50}$ 42 ± 11 vs 3.8 ± 1.6, p<0.05 vs GK) as well as high dose ET$_B$ blockade (EC$_{50}$ 42 ± 8.7 vs 3.8 ± 1.6, p<0.05 vs GK). Paradoxically, ET$_A$ blockade increased ET-1 sensitivity in Wistar rats. Maximum constriction to ET-1 was similar among groups. Vasorelaxation to ACh following 70% pre-constriction with 5-hydroxytryptamine (5-HT) was impaired in diabetes compared to control (Rmax 22 ± 4 vs 53 ± 7, p<0.05). ET$_A$ receptor blockade restored relaxation to control values in the GK animals (65 ± 4 vs 22 ± 4, p<0.05 vs GK) with no significant effect in Wistars. ET$_B$ blockade had no effect in either group. These findings
demonstrate the presence of cerebrovascular dysfunction in diabetes and suggest that the $\text{ET}_A$ receptor may exert a greater influence than the $\text{ET}_B$ in this process.
INTRODUCTION

Diabetes exists as an independent risk factor for cardiovascular disease (CVD) (1). Within the diabetic population, CVD is the leading cause of death accounting for 65-75% of all deaths (2). There is a strong correlation between diabetes and cerebrovascular disorders such as cerebral ischemia and stroke (3, 4). Diabetes not only increases the risk of stroke, but also contributes to increased stroke mortality and impaired recovery after stroke (5-7).

Regulation of vascular tone is important for maintenance of proper blood flow. In the cerebral circulation, changes in blood flow are buffered by the myogenic response to maintain cerebral blood flow. However, alterations to this system may be detrimental and could contribute to cerebrovascular disease. Previous studies have demonstrated increased myogenic tone in experimental diabetes (8-10). In addition to increased basal tone, cerebral arteries from diabetic animals exhibit diminished endothelium derived relaxation (8, 11, 12).

It is well established that the potent vasoconstrictor endothelin-1 (ET-1) is upregulated in the circulation in both clinical and experimental diabetes (13, 14). The effects of endothelin are mediated via two G-protein coupled receptors: ET$_A$ and ET$_B$ (15). ET$_A$ receptors reside on the smooth muscle cell (SMC) and produce vasoconstriction and mediate the proliferative effects of ET-1. Functional studies have suggested that different ET$_B$ receptor subtypes may exist (16-18). However, Mizuguchi et al demonstrated that the observed heterogeneous responses of the ET$_B$ receptor are abolished in ET$_B$ knockout mice suggesting that one gene is responsible for these actions (19). Cloning of the receptor will be the only definitive answer and to date, only
one ET\textsubscript{B} receptor has been cloned. Inasmuch, ET\textsubscript{B} receptors do elicit different responses. Nevertheless, some ET\textsubscript{B} receptors, designated ET\textsubscript{B1}, are on endothelial cells and promote vasodilation via cGMP while others, designated as ET\textsubscript{B2}, exist on smooth muscle cells (SMC) and educe ET\textsubscript{A}-like responses. Increasing evidence suggests that ET-1 is involved in the pathology of cerebrovascular disease (20). Studies have demonstrated enhanced contractile responses to ET-1 (21, 22) as well as a reduction of increased myogenic tone after ET receptor antagonism (8) in experimental diabetes. However, the relative roles of the endothelin receptors in Type-2 diabetic cerebrovascular dysfunction remain unknown. Therefore, the current study sought to determine the effects of chronic ET\textsubscript{A} or ET\textsubscript{B} receptor blockade on ET induced vasoconstriction and endothelium derived relaxation in basilar arteries from Type-2 diabetic (GK) rats.

**METHODS**

**Animals**

All experiments were performed on male Wistar (Harlan, Indianapolis, IN) and Goto-Kakizaki (GK, in-house bred, derived from the Tampa colony) rats (23, 24). The animals were housed at the Medical College of Georgia 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 until sacrifice at 18 weeks of age. Blood pressure was monitored either by telemetric method (as previously reported) (25).
or via the tail cuff method which we have previously validated on telemetry implanted animals.

**Endothelin receptor blockade**

At 14 weeks of age, animals were divided into groups and treated for four weeks as follows: *ET<sub>A</sub> receptor blockade* – ABT-627 (Abbott Labs) 5 mg/kg/day in drinking water, *ET<sub>B</sub> receptor blockade* – A-192621 (Abbott Labs) 15 or 30 mg/kg/day by oral gavage split into two daily doses or vehicle. Vehicle for the A-192621 consisted of 83% deionized water, 10% Polyethylene Glycol-400, 5% ethanol, and 2% Cremaphor EL. Tap water was used as vehicle for the ABT-627.

**Surgical procedure**

Animals were anesthetized with sodium pentobarbital and exsanguinated via cardiac puncture. The brain was quickly excised for isolation of basilar arteries.

**Determination of vascular function**

Isometric tension exerted by the vessels was recorded via a force transducer using the wire-myograph technique (Danish Myo Technologies, Denmark). The myograph chambers were filled with Kreb’s buffer (NaCl 118.3, NaHCO<sub>3</sub> 25, KCl 4.7, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, CaCl<sub>2</sub> 1.5 and Dextrose 11.1 mM), gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> and maintained at 37°C. Basilar artery segments (n=3-4/group, n=2 for high dose ET<sub>B</sub> due to mortality) were mounted in the chamber using 40 µm-thin wires and adjusted to a baseline tension of 0.5 g. Cumulative dose response curves to ET-1 (0.1-100 nM) were generated and the force generated was expressed as % change of baseline. Endothelium-dependent relaxation to acetylcholine (ACh, 1 nM-1 µM) was assessed after vessels were constricted to 60% of the baseline tension with serotonin.
(5-HT) or directly after ET-1 dose response. Sensitivity (EC$_{50}$) and maximum response ($R_{\text{max}}$) values were calculated from the respective dose-response equations.

**Hyperinsulinemic-euglycemic Clamp**

Overnight fasted animals (n=4/group) were placed under isoflurane anesthesia and placed on a heating pad to maintain body temperature. The carotid artery and femoral and jugular veins were exposed and cannulated with a PE 10 polyethylene tube. Glucose solution (100 mg/ml) and Insulin (NovoNordisk, 1U/ml) were connected to the femoral and jugular line respectively and the carotid clamped with hemostats to be used for blood glucose (BG) sampling. Two baseline glucose readings were drawn and the Insulin infusion was begun at a rate of 0.06 U/hr. Once euglycemia (125 mg/dl) was achieved, glucose infusion was started at a rate of 1 mg/min. Blood glucose readings were taken at five minute intervals for a total of 100 minutes and glucose infusion rate was adjusted at each interval to maintain euglycemia.

**Statistical analysis**

EC$_{50}$ and $R_{\text{max}}$ within-group differences were determined by $t$-tests. For multiple group comparisons, a one-way or two-way analysis of variance (ANOVA) was done accordingly, with a post hoc Bonferroni test. A repeated measures ANOVA was used to determine group differences (Diabetic vs. Control) across the ET-1 concentrations. Post-hoc group comparisons at each concentration used a Tukey’s adjustment for the multiple comparisons. Statistical significance was determined at p<0.05. Graphpad Prism 4.0 was used for all statistical tests performed.
RESULTS

Animal data

Metabolic parameters for control and GK rats animals are summarized in Table 4.1. Diabetic animals were significantly smaller than control animals and displayed mildly elevated blood glucose. Blood pressure was not significantly different between control and GK, but was slightly increased in control animals with high dose ET$_B$ blockade. Of note, some animals in the high dose (30 mg/kg/d) ET$_B$ blockade group developed lethargy and mild nose bleeding around week three of treatment. This effect was noted in both the diabetic and control animals. There was also increased mortality (a phenomena not previously seen in our studies) in these animals with 2 deaths in the control group and 1 in the diabetic group.

Hyperinsulinemic-euglycemic clamp studies demonstrated significantly impaired insulin sensitivity in the GK animals (Figure 4.1). Insulin resistance was determined as a decreased requirement of glucose to maintain euglycemia at the same rate of insulin infusion as control animals.

ET-1 dose-response

Basilar arteries of GK rats were hypersensitive to ET-1 (EC$_{50}$ 3.8 ± 1.6 nM) compared to control (13 ± 2 nM). (Figure 4.2A, Table 4.2). Blockade of the ET$_A$ receptor attenuated the contractile response of basilar arteries in diabetic animals as demonstrated by increased EC$_{50}$ values (3.8 ± 1.6 vs. 42 ± 11) (Figure 4.2B). Paradoxically, control animals exhibited increased sensitivity to ET-1 with ET$_A$ blockade (Table 4.2). Low dose ET$_B$ receptor blockade had no significant effect on ET-1 induced constriction in diabetic animals. However, high dose ET$_B$ blockade decreased sensitivity
similar to \( \text{ET}_A \) blockade (\( \text{EC}_{50} \ 42 \pm 8.7 \) vs \( 3.8 \pm 1.6 \) (GK Untreated)) (Figure 4.2B). There was no significant change in maximum constriction between all groups.

**Endothelium-dependent relaxation**

Basilar arteries of GK rats exhibited impaired endothelium relaxation following pre-constriction with serotonin (5-HT). Basilar arteries of GKS relaxed only \( 22 \pm 4\% \) in response to acetylcholine whereas the control rat arteries relax \( 53 \pm 7\% \). However, \( \text{ET}_A \) receptor blockade restored ACh induced relaxation to control values in the GK (\( 65 \pm 4\% \) vs. \( 53 \pm 7\% \), GK+ABT vs. Control) (Figure 4.3). There was no difference in maximum relaxation to acetylcholine in either of the groups. \( \text{ET}_B \) receptor blockade did not significantly affect endothelium dependent relaxation in 5-HT pre-constricted arteries at any dose, though there was a trend towards improved relaxation in the high dose \( \text{ET}_B \) blockade GKS (Figure 4.4A and B).

**DISCUSSION**

There are three major findings in the current study. First, basilar arteries were hypersensitive to ET-1 in a Type-2 diabetic rat model. Second, acetylcholine induced relaxation of basilar arteries was impaired in diabetes. Finally, these effects are primarily mediated by the \( \text{ET}_A \) receptor.

While there are many models of diabetes, most are either chemically induced Type-1 models or have co-morbid conditions such as hypertension, obesity and hyperlipidemia. The spontaneously diabetic Goto-Kakizaki is a non-obese, normotensive rat model originally developed from selective inbreeding of glucose intolerant Wistar rats (26). Previous studies have demonstrated that GK rats retain
greater than 40% of their beta cell mass and have a reduction of post-prandial glucose when given the insulin secretagogue nateglinide (27-30). In addition, our lab has previously shown that these rats have impaired glucose tolerance compared to control Wistars (25). In the current study, we have demonstrated that GK rats exhibit insulin resistance as assessed by the hyperinsulinemic-euglycemic clamp. Therefore, the GK rat serves as an excellent model for studying the effects of hyperglycemia in Type-2 diabetes.

We as well as others have observed elevated plasma ET-1 levels in both clinical and experimental diabetes (25, 31-33). Several studies have demonstrated enhanced contractile responses to ET-1 in aorta and peripheral arteries in diabetes (34-36). In addition, McIntyre et al reported increased sensitivity to ET-1 in subcutaneous resistance arteries from patients with Type-1 diabetes (37). In the present study, we have demonstrated an exacerbated contractile response of the rat basilar artery to ET-1 in diabetes. Matsumoto et al as well as Alabadi et al have both demonstrated similar results in the rat and rabbit basilar arteries respectively (21, 22). In contrast, Mayhan reported no differences in contractile responses to ET-1 in isolated rat basilar arteries (38). These discrepancies may be attributable to the differences in methodologies (e.g. in-vivo vs. in-vitro). Collectively, these previous studies were all performed in chemically induced Type-1 models of diabetes. To our knowledge, the present study is the first demonstration of enhanced cerebrovascular contractile response to ET-1 in a model of Type-2 diabetes.

Previous studies using models of Type-2 diabetes have demonstrated that endothelium derived relaxation is impaired in aortas and the peripheral vasculature (39-
42). However, Type-1 models, such as the streptozotocin rat and alloxan rabbit, have predominated in studies of the cerebral vessels (11, 12, 43, 44). Only in recent years have investigators begun to study the effects of Type-2 diabetes on the cerebral circulation and thus far, there are but a handful of these studies. Schwaninger and Karagiannis both reported diminished vasodilation in response to acetylcholine in obese Zucker rats (OZR) (45, 46). However, the OZR is a model of Type-2 diabetes known to have co-morbid conditions such as hypertension and hyperlipidemia which may contribute to altered vascular states. More recently, diabetic db/db mice, characterized by hyperinsulinemia, severe hyperglycemia and obesity, were shown to exhibit similar reductions in endothelium dependent relaxation (10). In the present study, we found that Type-2 diabetes significantly impairs acetylcholine induced relaxation in serotonin pre-constricted basilar arteries. ET$_A$ receptor antagonism completely restored ACh induced relaxation in diabetic basilar arteries indicating that an activated endothelin system contributes to this impairment. This data, in accordance with previous studies done in varying models of diabetes, suggests that diabetes impairs vascular relaxation without regard to the etiology of the disease or other co-morbid conditions.

The vascular effects of ET-1 are mediated by two distinct receptor subtypes: ET$_A$ and ET$_B$. In the present study, we have demonstrated hypersensitivity of the rat basilar artery to ET-1 in diabetes. ET$_A$ receptor blockade induced a significant rightward shift of the dose response curve indicating a decrease in sensitivity. Unexpectedly, higher dose blockade of the ET$_B$ receptor also produced a rightward shift of the dose response curve, in a manner similar to ET$_A$ blockade. Previous studies in diabetic rabbit and rat basilar arteries have produced similar results using in-vitro inhibition of ET$_B$ receptors.
Interestingly, Matsumoto et al showed that endothelial denudation of the diabetic basilar artery produced a significant leftward shift of the ET-1 dose response curve (21). Though not significant, we saw a trend to the same with lower dose ET\textsubscript{B} blockade possibly suggesting that removal of ET\textsubscript{B1} receptors alone affects the dilatory actions of ET in diabetes, while complete blockade (both ET\textsubscript{B1} and ET\textsubscript{B2}) alters vasoconstriction. ET-1 mediated constriction in the cerebrovasculature may be, in part, mediated through crosstalk of the ET receptor subtypes. Zuccarello et al demonstrated that ET\textsubscript{B} mediated vasoconstriction relies on activation of the smooth muscle as well as the endothelial ET\textsubscript{B} receptors (47).

Collectively, these studies strongly suggest a greater involvement of contractile ET\textsubscript{B2} receptors and/or an impairment of endothelial ET\textsubscript{B} in the development of vascular dysfunction in diabetes. Indeed, diabetes does upregulate vascular ET\textsubscript{B} receptor expression. Mumtaz et al found increased ET\textsubscript{B} receptor density in the diabetic rabbit urinary bladder while Ikeda showed a significantly increased ET\textsubscript{B} gene expression in STZ diabetic rat adrenal glands (48). In 10 week old NOD mice, a Type-1 diabetes model, aortic ET\textsubscript{B} gene expression was significantly increased while ET\textsubscript{A} expression was unchanged (49). Conceivably, blockade of ET\textsubscript{B} receptors, in a system which may be shifted more toward contractile responses, would produce effects similar to ET\textsubscript{A} blockade.

Though our studies correlate with previously published data, certain limitations must be addressed. First, we noted hyperreactivity of the basilar artery to ET-1. Due to limited availability of tissue in the GK rats, contractile responses to other vasoconstrictors were not measured and thus it is difficult to determine if this effect is
ET-1 specific. However, others have found similar responses to ET-1 in diabetic rat and rabbit basilar arteries (21, 22). Additionally contractile responses to UTP, serotonin, and the thromboxane analog U46619, were not different from control in these studies. Second, whether the effects of ET antagonism are a class effect or specific to the compounds used is indiscernible. Nonetheless, our data are consistent with other studies using in-vitro antagonists (BQ-123, BQ-788) (21, 22, 35). Future studies using varying constrictors and methods/compounds for inhibiting these receptors will better elucidate their functions in this model.

CONCLUSION

In summary, diabetes induces vascular dysfunction in isolated rat basilar arteries in the form of increased sensitivity to ET-1 and diminished relaxation capacity to acetylcholine. Inasmuch, these effects appear to be, at least in part, due to an increased contribution of the ET$_A$ / smooth muscle ET$_B$ receptor pathway. Alterations of cerebrovascular function may potentially underlie the diabetic propensity for cerebrovascular disease.
Acknowledgements

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REFERENCES


Table 4.1. Metabolic parameters of control Wistar and diabetic GK rats. *p<0.05 vs Wistar (n=4-5/group)

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|---------------------------------------------------------------|---------------------------------------------------------------|
| Wistar | Wistar + ABT-627 | Wistar + A-192621 15 mg/kg | Wistar + A-192621 30 mg/kg |
| Weight | 529 ± 18 | 535 ± 26 | 500 ± 13 | 406 ± 2* |
| Glucose | 110 ± 5 | 92 ± 0.9 | 109 ± 4 | 104 ± 5 |
| Insulin | 2.7 ± .55 | 2.1 ± .34 | 2.2 ± .53 | 2.2 ± .72 |
| ET-1 | 0.58 ± .16 | 0.37 ± .22 | 0.32 ± .18 | 1.6 ± .50* |
| MAP | 101 ± 2 | 96 ± 5 | 102 ± 11 | 122 ± 3* |
| Cholesterol | 66 ± 2 | 76 ± 7 | 67 ± 3 | 72 ± 8 |
| Triglycerides | 65 ± 9 | 50 ± 11 | 50 ± 9 | 81 ± 9 |

| GK | GK + ABT-627 | GK + A-192621 15 mg/kg | GK + A-192621 30 mg/kg |
| Weight | 365 ± 13* | 370 ± 12* | 334 ± 17* | 322 ± 10* |
| Glucose | 272 ± 58* | 202 ± 9* | 304 ± 17* | 173 ± 31* |
| Insulin | 0.96 ± .25* | 0.66 ± .12* | 0.90 ± .15* | 0.64 ± .10* |
| ET-1 | 1.3 ± .2* | 1.2 ± .07* | 1.4 ± .17* | 0.88 ± .07 |
| MAP | 110 ± 7 | 107 ± 5 | 104 ± 17 | 116 ± 2* |
| Cholesterol | 80 ± 4 | 77 ± 3 | 81 ± 7 | 86 ± 9 |
| Triglycerides | 30 ± 2* | 22 ± 2* | 22 ± 4* | 39 ± 12 |
TABLE 4.2

Table 4.2. Sensitivity (EC$_{50}$) and magnitude (Rmax) of vascular responses to ET-1 and ACh in the absence or presence of ET receptor antagonists in basilar arteries of control Wistar and diabetic Goto-Kakizaki rats. *p<0.05 vs Wistar, **p<0.05 vs GK

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**FIGURE LEGENDS**

**Figure 4.1.** Hyperinsulinemic-euglycemic clamp in diabetic GK and control Wistar rats.  
A. Plasma glucose measurements at 5 minute intervals demonstrate that euglycemia (~125 mg/dl) was maintained from approximately 40 to 110 minutes.  
B. GK rats were less sensitive to insulin as indicated by decreased requirements for glucose during continuous, fixed insulin infusion.  
* p<0.05 vs control

**Figure 4.2.** Constriction responses to ET-1 in basilar arteries of diabetic GK and control Wistar rats.  
A. Compared to Wistars, GK rats exhibited increased sensitivity (EC$_{50}$) to ET-1, although the magnitude of constriction did not differ.  
B. ET$_A$ receptor antagonism decreased ET-1 sensitivity in GKs as demonstrated by a rightward shift of the dose response curve and increased EC$_{50}$. There was no significant difference with low dose ET$_B$ blockade. However, high dose ET$_B$ blockade caused a rightward shift of the dose response almost identical to that caused by ABT-627.  
* EC$_{50}$ p<0.05 vs control, ** EC$_{50}$ p<0.05 vs GK.

**Figure 4.3.** Relaxation responses to acetylcholine in basilar arteries from diabetic GK and control Wistar rats. GK rats had impaired endothelium dependent relaxation as compared to controls. ET$_A$ receptor blockade restored maximum ACh induced relaxation in GK basilar arteries to greater than control values.  
* R$_{max}$ p<0.05 vs control, **R$_{max}$ p<0.05 vs GK
**Figure 4.4.** Effects of ET$_B$ receptor blockade on ACh induced relaxation in diabetic (GK) and control (Wistar) rats. **A. & B.** There was no significant effect of either low or high ET$_B$ receptor blockade on ACh induced relaxation of basilar arteries in GK or Wistar rats.
FIGURE 4.1

Plasma Glucose

A.

BG (mg/dl)

0 10 20 30 40 50 60 70 80 90 100 110

Time (min)

- GK
- Wistar

Glucose Infusion Rate

B.

GINFR (mg/min)

0 5 10 15

Time (min)
FIGURE 4.2

A.

B.
FIGURE 4.3

% Relaxation of 5-HT preconstriction

log [ACh]
FIGURE 4.4

A. Wistar
* Wistar + A-192621 15 mg/kg
X Wistar + A-192621 30 mg/kg

B. GK
▼ GK + A-192621 15 mg/kg
◆ GK + A-192621 30 mg/kg

log [ACh] % Relaxation of 5-HT preconstriction

% Relaxation of 5-HT preconstriction

log [ACh]
CHAPTER 5

ET$_B$ RECEPTOR ANTAGONISM ATTENUATES CEREBROVASCULAR REMODELING IN TYPE-2 DIABETES$^1$

$^1$Harris, A.K., Sachidanandam, K., Hutchinson, J., Ergul, A. Under preparation to be submitted to *Atherosclerosis Thrombosis and Vascular Biology*
ABSTRACT

Diabetes enhances vascular matrix accumulation, which can further contribute to the development of vascular dysfunction. Endothelin-1 (ET-1) may contribute to this process by stimulation of collagen synthesis and smooth muscle cell (SMC) migration. We have previously demonstrated that blockade of the ET\textsubscript{A} receptor attenuates dysregulation of matrix degrading enzymes (MMP) and blunts collagen deposition and matrix remodeling in Type-2 diabetes. A recent study demonstrated that inhibition of the ET\textsubscript{B} receptor by pharmacological or genetic approaches promotes pathological vascular remodeling in a vascular injury model suggesting that ET\textsubscript{B} receptors are vasculoprotective. However, the relative role of the ET\textsubscript{B} receptor in Type-2 diabetic cerebrovascular remodeling processes and its influence on the vascular matrix remained unknown. Accordingly, the current study tested the hypothesis that pharmacological inhibition of the ET\textsubscript{B} receptor augments vascular remodeling of middle cerebral arteries in Type-2 diabetes.

Fourteen week old Goto-Kakizaki rats were treated for 4 weeks with the ET\textsubscript{B} receptor antagonist A-192621 (15 or 30 mg/kg/day). High dose receptor blockade significantly reduced MMP-2 protein (44 ± 15* vs 201 ± 13 pixels) and activity (12151 ± 1004* vs 18296 ± 1180 pixels) in diabetic middle cerebral arteries while lower dose inhibition had no significant effect. Morphometric analysis of cross sections demonstrated that similar to MMP-2 protein changes, low dose receptor blockade had no effect on vessel structure. However, high dose blockade significantly reduced wall:lumen ratio (0.23 ± .03* vs 0.51 ± .04) in a manner similar to that observed with ET\textsubscript{A} receptor blockade. These data indicate a potentially greater involvement of the smooth
muscle ET$_{B2}$ receptor in Type-2 diabetes giving rise to ET$_{A}$-like effects in the vasculature as opposed to anticipated vasculoprotective effects via activation of ET$_{B1}$ receptors on endothelial cells. These findings also point to a potential receptor interaction among ET$_{A}$, ET$_{B1}$ and ET$_{B2}$ subtypes and may be relevant for the future development and use of ET receptor antagonists in diabetes.
INTRODUCTION

The extracellular matrix (ECM) is a dynamic scaffold with the purpose of maintaining the integrity of blood vessels as well as other tissues. When compromised, the delicate homeostasis of protein synthesis/degradation becomes disrupted and educes pathological changes in the vasculature. These changes are often associated with vascular complications seen in diabetes (1, 2). However, the mechanistic regulation of these events and how they are altered by diabetes is not well understood.

Hypertrophic remodeling is observed with both human and experimental diabetes and in both the micro and macrovasculature (3-5). We have previously demonstrated that increased media to lumen ratio in middle cerebral arteries (MCA) from Type-2 diabetic rats is associated with dysregulation of matrix metalloproteinase (MMP) activity (6). MMPs are a family of zinc dependent proteases that, collectively, regulate the turnover of various ECM proteins (7, 8). MMPs affect the matrix not only through degradation but also through the release of cryptic signals and growth factor activation which can in turn stimulate collagen synthesis and deposition (9, 10).

The potent vasoconstrictor ET-1, which is elevated in human and experimental diabetes, has both vasoactive and mitogenic properties that are carried out by its receptors (6, 11). ET_a receptors located on vascular smooth muscle cells (VSMC) promote vasoconstriction and cellular proliferation in many cell types. ET_b receptors have a putative role of being primarily vasodilatory as well as vasculoprotective (12). However, these receptors produce heterogeneous responses depending on their localization (13). ET_B1 receptors, located on endothelial cells, do indeed elicit vasodilation via cGMP. However ET_B2 receptors, located on VSMC, produce effects
similar to the ET\textsubscript{A} receptors. Increasing evidence suggests that the balance of these receptors within the vasculature may favor the contractile ET\textsubscript{B2} phenotype under pathological circumstances (14-19). We have previously demonstrated that chronic blockade of the ET\textsubscript{A} receptor in Type-2 diabetes decreases vascular remodeling by attenuation of MMP dysregulation (6). However, whether and to what extent the ET\textsubscript{B} receptor(s) contribute to this process is unknown. Accordingly, the current study sought to determine the relative involvement of the ET\textsubscript{B} receptor(s) in Type-2 diabetic cerebrovascular remodeling using specific pharmacological receptor inhibition.

**METHODS**

**Animal Studies**

The Medical College of Georgia Institutional Animal Care and Use Committee (IACUC) approved all protocols. Male Wistar (Harlan, Indianapolis, IN) and Goto-Kakizaki (in-house bred, derived from the Tampa colony) rats were used for all studies. All animals were individually housed at the Medical College of Georgia’s animal care facility, were allowed access to food and water ad libitum, and were maintained on a 12/12 hour light/dark cycle. During housing, weight, and blood glucose measurements were performed twice weekly. Glucose measurements were taken from the tail vein and measured on a commercially available glucose meter (AccuChek, Roche Diagnostics, Indianapolis, IN). At 14 weeks of age, animals were administered the ET\textsubscript{B} selective antagonist, A-192621 (15 or 30 mg/kg/day), or vehicle only. Vehicle for the A-192621 consisted of 83% deionized water, 10% polyethylene glycol-400, 5% ethanol, and 2% Cremaphor EL. Animals were dosed twice daily by oral gavage with either half of their
total daily dose of drug or vehicle alone. Treatment was maintained until the time of
sacrifice at 18 weeks of age. Animals were anesthetized with sodium pentobarbital and
exsanguinated via cardiac puncture. Upon sacrifice, the brain was removed and placed
in ice-cold physiological saline solution. For immunohistochemistry, middle cerebral
arteries (MCA) were perfused with Histogel (Richard Allen Scientific, Kalamazoo, MI),
then excised and embedded in the same matrix. Upon gelling of the matrix, the
embedded vessel was placed in 10% formalin for storage. For protein studies, vessels
were excised, snap frozen in liquid nitrogen, and stored at -80°C.

**Plasma Measurements**

Plasma insulin was measured using enzyme-linked immunosorbant assay kits
(Alpco Diagnostics, Windham, NH). Plasma triglycerides and cholesterol were
estimated using commercial kits (Wako, Richmond, VA).

**Tissue Homogenization and Gelatin Zymography**

Snap-frozen MCAs were homogenized and gelatin zymography was performed
on them as previously described (6). Gelatinolytic activity was assessed by
densitometric analysis (Gel-Pro version 3.1, Media Cybernetics, Carlsbad, CA). Tissue
inhibitor of metalloproteinase-2 (TIMP-2) levels were measured by enzyme-linked
immunosorbent assay (Amersham Biosciences, Piscataway, NJ).

**Immunohistochemistry and Vessel Morphometry**

Middle cerebral arteries were processed for immunohistochemistry as described
previously (6). For morphometric analysis, 4 µm vessel segments were stained with
Masson’s trichrome stain. Slides were viewed using an Axiovert microscope (Zeiss,
Thornwood, NY), and images were captured and analyzed using Spot software for determination of media-to-lumen ratios (M/L).

**Immunoblotting**

Protein levels of MMP-2 were measured by Western blotting as previously described (6). All blots were stripped and re-probed with anti-actin antibody to ensure equal protein loading.

**RESULTS**

**Animal Data**

Metabolic parameters for control and diabetic (GK) animals are summarized in Table 5.1. Diabetic animals were significantly smaller than control. High dose receptor blockade caused a reduction of body weight in control and, to a lesser degree, diabetic animals. GK animals displayed mildly elevated blood glucose that was slightly reduced by high dose receptor blockade. There was no difference in blood pressure or lipids between untreated control and diabetic animals. Treatment did not significantly affect blood pressure in the diabetic animals, however, high dose blockade significantly elevated BP in the control animals. Plasma insulin levels were significantly lower in GK animals but we and others have previously demonstrated that, at 18 weeks of age, this model exhibits insulinopenia and insulin resistance (6). Treatment had no significant effect on insulin levels, triglycerides, or cholesterol in any group.

**Morphology of Middle Cerebral Arteries**

Diabetic animals exhibited increased media thickness of MCAs with a significantly increased media:lumen ratio (p<0.001). Low dose blockade failed to
produce any significant effect. However, high dose blockade significantly attenuated medial thickening in diabetes and reduced media:lumen ratios to control levels ($p<0.001$ vs GK). Treatment had no effect on medial thickness of control animals. Representative images for each group are shown in Figure 5.1A and media:lumen ratios summarized in Figure 5.1B.

**MMP Protein Expression and Activity**

Total MMP-2 expression was assessed in MCAs by immunoblotting. Total MMP-2 protein was slightly higher in diabetic animals as compared to controls and low dose receptor antagonism had no effect on MMP protein levels. However, high dose blockade significantly reduced MMP-2 protein in diabetic animals with no effect on controls (Figure 5.2A). Gelatinase activity was assessed by zymography and no difference in lytic activity was detected between control and diabetic animals. High dose receptor blockade reduced diabetic MMP-2 activity to less than control while low dose had no effect (Figure 5.2B). TIMP-2 levels were assessed in MCAs by ELISA. Control and diabetic TIMP-2 levels were not different. High dose blockade slightly reduced diabetic TIMP-2 levels with a trend for decreased TIMP-2 in controls (Figure 5.2C). Representative immunoblots and zymograms are shown in Figure 5.2.

**DISCUSSION**

Maintenance of extracellular matrix homeostasis is an important event to ensure proper structure and function of vessels. Numerous studies have demonstrated medial hypertrophy and increased media:lumen ratios in the peripheral vessels in diabetes (4, 20-22). However, much less is known about the effects of diabetes on the
cerebrovasculature. We have previously demonstrated that experimental Type-2 diabetes induces vascular remodeling resulting in increased media:lumen ratios in middle cerebral arteries. Furthermore, \( \text{ET}_A \) receptor blockade attenuated medial hypertrophy in this model, implicating a role for ET-1 in the regulation of this process (6). Therefore, the current study was designed to further elucidate the ET system in this process by assessing the relative role of the \( \text{ET}_B \) receptors in this vascular bed.

The vasoconstrictor peptide ET-1 has been implicated in many disease states (23-25) and previous studies have demonstrated that ET-1 influences ECM dynamics in diabetes. In diabetic animals, ET-1 receptor antagonism has been reported to prevent ECM deposition in the retina as well as renal arteries (20, 26). Recently, Amiri et al have demonstrated that endothelium restricted overexpression of ET-1 causes marked hypertrophic remodeling and endothelial dysfunction in mice (27). Similar to our previous results, Spiers et al reported attenuation of increased vascular MMP-2 activity following ET antagonism (28) while others have shown that treatment with the \( \text{ET}_A \) selective antagonist, Sitaxsentan, reduces post MI left ventricular dilation as well as MMP activity in cardiac tissue (29). These observations indicate a putative role for ET in the regulation of MMPs. In contrast to our previous findings, the present study did not detect significant differences in MMP-2 activity in MCAs from control and diabetic animals although total MMP-2 protein was higher in diabetes (in accordance with our previous results). High dose receptor blockade reduced MMP-2 protein and slightly reduced MMP-2 activity in diabetes while the low dose had no effect. This discrepancy in basal MMP activity could be due to variation in the data, possible influences by the
drug administration route (gavage vs drinking water), or limitations of the zymographic technique to detect subtle differences.

We hypothesized that ET$_B$ receptor antagonism would block the vasculoprotective effects of endothelial ET$_B$ receptors and would exacerbate vascular remodeling in diabetes. Conversely, we found that low dose receptor blockade failed to effect a difference in media:lumen ratio compared to untreated GKs while high dose ET$_B$ blockade significantly reduced medial hypertrophy and decreased media:lumen ratio to control values. This was completely unexpected based on previous reports of enhanced neointimal hyperplasia and medial thickening (12) and increased aortic wall thickness and media:lumen ratios (30) following genetic deletion or pharmacological inhibition of ET$_B$ receptors. However, emerging evidence suggests that differential roles for the ET$_B$ receptor may exist.

The vascular effects of ET-1 are mediated by two receptor subtypes: ET$_A$ and ET$_B$. ET$_A$ receptors are located on smooth muscle cells and mediate vasoconstriction and proliferation while ET$_B$ receptor locations/actions vary. ET$_{B1}$ receptors located on endothelial cells mediate relaxation via cyclic GMP while ET$_{B2}$ receptors are located on SMC and behave as ET$_A$s. It has been demonstrated that diabetes upregulates vascular ET$_B$ receptor expression. Mumtaz et al found increased ET$_B$ receptor density in the diabetic rabbit urinary bladder while Ikeda showed a significantly increased ET$_B$ gene expression in STZ diabetic rat adrenal glands (19, 31). Most notably, contractile ET$_{B2}$ receptors are upregulated in rat MCAs following focal cerebral ischemia (32). This apparent change of receptor balance has been noted in functional studies as well. Numerous studies in experimental diabetes have demonstrated attenuation of ET-1
hyperreactivity in cerebral arteries following ET\textsubscript{B} receptor blockade (14-16) producing effects similar to those observed with ET\textsubscript{A} antagonism. It is clear that the distribution and/or expression of ET receptors in the vasculature is altered in pathological states such as diabetes. In view of the current study, it is plausible that low dose receptor blockade was incomplete and failed to modify the vascular structure while higher dose antagonism blocked ET\textsubscript{A}-like ET\textsubscript{B2} receptors, thus reducing media:lumen ratios. Indeed, MMP-2 protein, which has been shown to stimulate collagen synthesis via transactivation, was significantly decreased in diabetes. However, future experiments will be required to clarify this process.

Future experiments will include receptor binding studies to determine the relative density and expression of ET receptors in the cerebral vessels. Additionally, more animals will be added, and possibly, a different route of drug delivery will be employed to reduce variability in the samples (i.e. protein expression and activity). Other potential studies which would help elucidate this system better would be in-vitro vascular function studies utilizing different ET antagonists and/or endothelial removal as well as in-vivo studies using various ET receptor blockers, including RES 701-1, a purported endothelial ET\textsubscript{B1} selective antagonist. Nevertheless, the current study has demonstrated that ET\textsubscript{B} receptor antagonism with A-192621, 30 mg/kg/d, significantly attenuates vascular remodeling in middle cerebral arteries from Type-2 diabetic rats.
Acknowledgements

This study was supported by research awards from NIH (HL076236, DK074385), American Diabetes Association and Philip Morris Foundation to Adviye Ergul and AHA Southeast Affiliate Pre-Doctoral Fellowship to Alex K. Harris.
REFERENCES


26. Evans, T., et al., Endothelin receptor blockade prevents augmented extracellular matrix component mRNA expression and capillary basement


**TABLE 5.1**

*Table 5.1. Metabolic parameters of control Wistar and diabetic GK rats. *p<0.05 vs. Wistar*

<table>
<thead>
<tr>
<th></th>
<th>Wistar</th>
<th>Wistar + A-192621 15 mg/kg</th>
<th>Wistar + A-192621 30 mg/kg</th>
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<tr>
<td>Weight</td>
<td>529 ± 18</td>
<td>500 ± 13</td>
<td>406 ± 2*</td>
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<tr>
<td>Glucose</td>
<td>110 ± 5</td>
<td>109 ± 4</td>
<td>104 ± 5</td>
</tr>
<tr>
<td>Insulin</td>
<td>2.7 ± .55</td>
<td>2.2 ± .53</td>
<td>2.2 ± .72</td>
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<tr>
<td>ET-1</td>
<td>0.58 ± .16</td>
<td>0.32 ± .18</td>
<td>1.6 ± .50*</td>
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<tr>
<td>MAP</td>
<td>101 ± 2</td>
<td>102 ± 11</td>
<td>122 ± 3*</td>
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<tr>
<td>Cholesterol</td>
<td>68 ± 2</td>
<td>67 ± 3</td>
<td>72 ± 8</td>
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<tr>
<td>Triglycerides</td>
<td>65 ± 9</td>
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<table>
<thead>
<tr>
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<th>GK</th>
<th>GK + A-192621 15 mg/kg</th>
<th>GK + A-192621 30 mg/kg</th>
</tr>
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<tbody>
<tr>
<td>Weight</td>
<td>365 ± 13*</td>
<td>334 ± 17*</td>
<td>322 ± 10*</td>
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<tr>
<td>Glucose</td>
<td>272 ± 58*</td>
<td>304 ± 17*</td>
<td>173 ± 31*</td>
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<tr>
<td>Insulin</td>
<td>0.96 ± .25*</td>
<td>0.90 ± .15*</td>
<td>0.64 ± .10*</td>
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<td>ET-1</td>
<td>1.3 ± .2*</td>
<td>1.4 ± .17*</td>
<td>0.88 ± .07</td>
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<tr>
<td>MAP</td>
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<td>Cholesterol</td>
<td>80 ± 4</td>
<td>81 ± 7</td>
<td>86 ± 9</td>
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<tr>
<td>Triglycerides</td>
<td>30 ± 2*</td>
<td>22 ± 4*</td>
<td>39 ± 12</td>
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</table>
FIGURE LEGENDS

Figure 5.1. A. Representative cross sections of MCAs demonstrating vascular remodeling in diabetes. B. Summary of wall:lumen ratios in diabetic and control MCAs with and without ET$_B$ receptor antagonism. *p<0.05 vs Wistar, **p<0.05 vs GK

Figure 5.2. MMP protein expression and activity in MCAs from diabetic and control MCAs. A. MMP-2 protein was slightly elevated in diabetes. High dose ET$_B$ blockade significantly reduced MMP-2 protein to control levels. Representative immunoblot is shown below the histogram. B. MMP-2 activity was not different in diabetes. High dose blockade slightly reduced diabetic MMP-2 activity to less than control. C. TIMP-2 protein levels, as assessed in MCAs by ELISA, were not different in diabetes. High dose ET$_B$ receptor antagonism slightly reduced TIMP-2 levels in GKS. *p<0.05 vs Wistar, **p<0.05 vs GK
FIGURE 5.1

A. Untreated A-192621 A-192621
15 mg/kg/d 30 mg/kg/d

Wistar

GK

GK + A-192621 Low
GK + A-192621 High
Wis
Wis + A-192621 Low
Wis + A-192621 High

B. Wall:Lumen Ratio

GK
GK + A-192621 Low
GK + A-192621 High
Wis
Wis + A-192621 Low
Wis + A-192621 High
FIGURE 5.2

A. MMP-2 Protein OD (pixels)

B. MMP-2 Activity OD (pixels)

C. TIMP-2 ng/mg protein

- GK
- GK + A-192621 15 mg/kg
- GK + A-192621 30 mg/kg
- Wistar
- Wistar + A-192621 15 mg/kg
- Wistar + A-192621 30 mg/kg
CHAPTER 6
CONCLUDING REMARKS

In September 2005, three specific aims were proposed to determine the effects of diabetes and the influence of the endothelin system on cerebrovascular structure and MMPs in Type-2 Goto-Kakizaki rats. These aims have been modified in this dissertation to include an investigation of cerebrovascular function in this model as well (see Dissertation Objectives, Chapter 1). The proposed experiments to accomplish this, as well as the vascular function experiments, have been completed and compiled in manuscript form (two of which have been published). The major findings of these studies are summarized below.

- **Characteristics of the Goto-Kakizaki Rat:** Our studies revealed that the GK rat exhibits mild hyperglycemia, insulinopenia, and marked insulin resistance at 18 weeks of age. Plasma ET-1 levels were significantly increased while total cholesterol, triglycerides, and blood pressure were not different from control.

- **Vascular Structure in Type-2 Diabetes:** Morphometric analysis of MCAs from diabetic animals revealed a significant increase in media:lumen ratio, manifested as medial hypertrophy without a change in overall vessel size. $\text{ET}_A$ receptor antagonism slightly, but significantly, attenuated MCA media:lumen ratio. Contrary to the expected outcome, $\text{ET}_B$ receptor blockade also significantly reduced
vascular remodeling, but to a much greater degree than ET$_A$ blockade. This data suggests a greater participation of contractile and proliferative ET receptors in the regulation of vascular ECM dynamics.

- **Vascular MMP Expression:** MMP-1 (collagenase) was significantly decreased in diabetic MCAs while MMP-2 (gelatinase) protein and activity were increased. ET$_A$ receptor blockade increased MMP-1 and decreased MMP-2 to control levels while attenuating vascular remodeling. ET$_B$ antagonism significantly reduced MMP-2 protein and slightly reduced MMP-2 activity in MCAs. These results will be confirmed in future studies.

- **Contractile Response to ET-1:** Cumulative dose response curves revealed that basilar arteries from diabetic animals were hypersensitive to ET-1 as indicated by reductions of the EC$_{50}$. However, maximal responses were not different. Blockade of the ET$_A$ receptor produced a rightward shift of the dose response curve and decreased sensitivity, consistent with blockade of contractile actions. However, high dose ET$_B$ blockade produced a nearly identical curve to that seen with ET$_A$ antagonism. Interestingly, low dose blockade of the ET$_B$ receptors increased sensitivity, (though not significant) similar to others results with endothelial denudation.

- **Endothelium Dependent Relaxation:** Compared to controls, diabetic animals exhibited significantly impaired relaxation in response to acetylcholine in 5-HT pre-constricted basilar arteries. ET$_A$ antagonism
significantly attenuated this affect and restored vasorelaxation to a level better than control. However, there was no effect of ET$_B$ receptor blockade on ACh induced relaxation. NO blockade with the NOS inhibitor LNNA did not further impair endothelium-mediated relaxation suggesting that the NOS system may be dysfunctional in Type-2 diabetic cerebrovasculature.

Vascular structural changes influence function by altering distensibility, elasticity, and compliance of blood vessels. In addition, disruption or modification of the extracellular matrix can uncover cryptic signals which in turn can begin a signal cascade leading to further cell proliferation and migration. Data presented within this dissertation demonstrate that Type-2 diabetes, devoid of confounding conditions such as hypertension and hyperlipidemia, induces structural as well as functional changes in the cerebrovasculature which are at least modulated, if not regulated by the endothelin system. Diabetes is a monumental disease with nearly 75,000 deaths per year, mostly from cardiovascular complications, in the United States alone. Though seemingly small in the big scheme of things, these studies and others like them will hopefully set the footings upon which novel therapeutic interventions can be developed.

**Future Directions**

Future studies are necessary to better elucidate the mechanisms by which diabetes alters vascular homeostasis. The effects of hyperglycemia and glycemic control need to be investigated utilizing methods to control blood glucose as well as potentially performing similar studies in different models of diabetes. Additionally, more
thorough examination of the ET system is a must to determine the discreet actions of the various receptor subtypes. These studies could include in-vitro cell culture models, receptor deficient animals, as well as utilization of various other pharmacological inhibitors of ET receptors. Furthermore, additional studies to assess the origin of vascular dysfunction in this vascular bed need to be done. Specifically, additional vasoconstrictors, vasodilators, as well as blockade of other pathways (i.e. NO, COX, etc.) would better clarify the etiology of dysfunction and strengthen previous studies.