THE RELATIONSHIP OF VASCULAR STRUCTURE, MECHANICS AND FUNCTION

IN RESISTANCE ARTERIES IN TYPE 2 DIABETES

by

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(Under the Direction of Adviye Ergul)

ABSTRACT

Diabetes leads to restructuring of vascular extracellular matrix components, resulting in increased media-to-lumen (M/L) ratios, decreased vascular compliance, and decreased relaxation in microvessels, thus increasing cardiovascular risk. This study examined individual and combined roles of hyperglycemia in Type 2 diabetes and oftentimes accompanying co-morbid factor, hyperlipidemia, in mediating mesenteric microvascular remodeling, impaired mechanical properties and vascular dysfunction in Type 2 diabetes. Increased vascular M/L ratios were observed in diabetes that were normalized by glycemic control with metformin, and combined hyperglycemia and hyperlipidemia did not cause further worsening of vessel morphology. Glycemic control also decreased vascular stiffness and myogenic tone in diabetes, thus improving compliance. In addition to worsening cardiometabolic factors, combined hyperglycemia and hyperlipidemia had adverse effects in mediating vascular dysfunction. Thus, by understanding the relative roles of diabetes and obesity, it is beneficial in devising effective therapeutic strategies to treat microvascular complications in diabetes.

Index words: Type 2 diabetes, hyperlipidemia, vascular structure, vascular compliance, vascular function, extracellular matrix, animal models.

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CHAPTER 1

INTRODUCTION

A. Statement of the problem and dissertation objectives.

Nearly 21 million people in the United States have diabetes, which accounts to about 7 percent of the total population. While two-thirds of them have been diagnosed, a significant one-third from this group remains undiagnosed. Adults with diabetes are at a two to four-fold higher risk of heart disease and stroke that comprises about 65 percent of all diabetes-related deaths. Diabetes is the leading cause of high blood pressure, blindness, kidney disease, nervous system damage and lower-limb amputations. It is also a major cause of concern in dental health and pregnancy. The total estimated costs of diabetes management in the United States totaled to $132 billion in 2002, thus posing immense burden on the health-care system.

Type 2 diabetes accounts for about 90 to 95 percent of all diagnosed cases of diabetes. It was previously called non-insulin dependent diabetes mellitus (NIDDM), which usually begins as insulin resistance, a condition wherein the cells are unable to utilize the secreted insulin and gradually results in deficient insulin production by the pancreatic islet cells. Thus, sensitization of the cells in the body to the insulin that is produced, or facilitating the pancreatic beta cells to produce more insulin have been frontline therapeutic approaches in treating Type 2 diabetes. However, glycemic control,

a powerful strategy in diabetes management has not been used to potential partly because most clinical trials demonstrating its beneficial effects have been shown in patients with Type 1 diabetes, which is of a completely different etiology. Another reason was because of the paucity of pre-clinical studies in Type 2 diabetes to back clinical data, due to the lack of a relevant animal model. The Goto Kakizaki (GK) rat is an excellent model to study Type 2 diabetes, because of its pathological similarity to the humans with respect to the onset and progression of the disease. With mild to moderate levels of blood glucose (150 – 200 mg/dl) it closely mimics glycemic profiles of patients with the condition.

Type 2 diabetes is also commonly accompanied by co-morbid obesity, hyperlipidemia, hypertension and insulin resistance. This cluster of conditions is referred to as the metabolic syndrome or syndrome X. With its multifaceted pathology, it becomes a challenge from a therapeutic standpoint. Pre-clinically, several models have been successfully developed to study the effects of metabolic syndrome. The Zucker obese rat and the db/db mouse are the most commonly used models, which present all the components of the syndrome. However, in order to understand relative contributions of these conditions, it is important to dissect their role. The individual as well as the combined roles of Type 2 diabetes and hyperlipidemia can be studied in the GK rat by administration of a high-fat diet.

Changes in vascular function, such as increased constriction or decreased relaxation to vasoactive agents, alterations in vascular structure such as medial thickening or intimal hyperplasia or impaired mechanical properties and decreased compliance of microvessels contribute to increased cardiovascular risk in Type 2

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diabetes. However, the relative contributions of hyperglycemia and hyperlipidemia in mediating these manifestations, or the role of glycemic control in protecting from or delaying vascular pathology are not well understood.

Thus, we hypothesized that hyperglycemia-induced microvascular dysfunction as well as remodeling would be exacerbated by hyperlipidemia via differential regulation of matrix metalloproteases, key enzymes responsible for extracellular (ECM) matrix turnover. Our goal was to demonstrate that glycemic control would improve vascular function and decrease remodeling, whereas, hyperlipidemia would potentiate vascular structural, mechanical and functional anomalies in diabetes. We proposed to examine the mesenteric microvasculature as a model for systemic vascular remodeling in Type-2 diabetes.
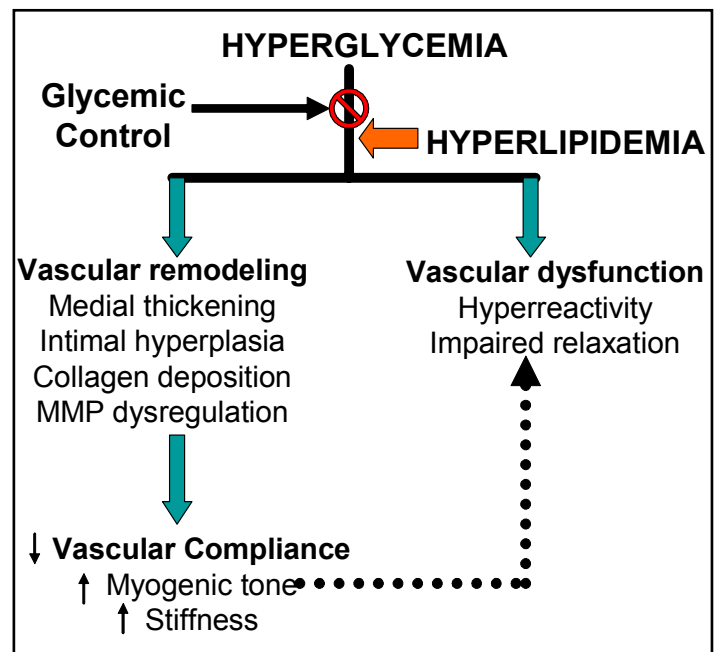
Figure 1.1. Scheme outlining the proposed hypotheses

These hypotheses were tested in two specific aims using the Goto-Kakizaki (GK) rat, a non-obese, normotensive and spontaneous model of Type-2 diabetes. Control (Wistar) rats, diabetic and euglycemic GKs, high-fat fed controls and GKs were used for the proposed studies.

Specific Aim 1: To test the hypothesis that hyperlipidemia would exacerbate hyperglycemia-mediated vascular remodeling by differentially augmenting expression and activity of MMP isoforms. Two sub-studies were designed to address this aim.

Sub-study 1a). To what degree does hyperglycemia contribute to vascular remodeling and decreased compliance, and what is the role of glycemic control in attenuating these pathological outcomes?

Sub-study 1b). To what degree does hyperlipidemia exacerbate structural remodeling and impaired vascular compliance of resistance microvessels in Type-2 diabetes?

- Structural changes were determined in mesenteric microvessels by morphometric analysis and quantification of extra-cellular matrix (ECM) proteins - collagen and fibronectin by histochemical and biochemical approaches.

- Expression and activity of MMPs and tissue inhibitors of MMPs (TIMPs) were determined by immunoblotting and gelatin zymography.

Based on current literature, our prediction was that hyperglycemia alone would promote medial thickening or extracellular matrix deposition via activation of MMP-2 and hyperlipidemia would augment MMP-9 activity.

Specific Aim 2: To test the hypothesis that hyperlipidemia would increase vasoconstriction mediated by hyperglycemia and decrease compliance of resistance arteries.

Sub-study 2a). To what degree does hyperglycemia contribute to vascular dysfunction and decreased compliance, and what is the role of glycemic control in attenuating these pathological outcomes?

Sub-study 2b). What role does diet-induced hyperlipidemia play in altering vascular mechanics and intensifying vascular dysfunction in diabetes?

- Distensibility, stiffness and myogenic tone were assessed in isolated and pressurized mesenteric microvessels using a perfused arteriograph system.
- Constriction and relaxation responses to vasoactive agents were quantified using the arteriograph system.

Our expectation was to see decreased distensibility, increased stiffness and myogenic tone in hyperglycemia, worsened with combined hyperlipidemia. We anticipated that hyperreactivity to vasoconstrictors and impaired relaxation responses in Type-2 diabetes will be exacerbated with a high-fat diet.

B. Brief review of literature and discussion of the rationale of the project.

Type 2 Diabetes

Diabetes is a condition marked by high levels of blood glucose resulting from defects in insulin production, insulin action, or both. It can lead to serious complications and premature death, but by controlling diabetes, these can be minimized (1; 2). It is estimated that over 18 million Americans are affected by Type 2 diabetes, which accounts for about 90 to 95 percent of all diagnosed cases of diabetes. It usually begins as insulin resistance, a disorder in which the cells do not utilize insulin properly. As the need for insulin rises, the pancreatic cells gradually lose the ability to produce it. Type 2 diabetes is associated with ageing, obesity, family history of diabetes, a history of gestational diabetes, impaired glucose metabolism, physical inactivity and race/ethnicity. African Americans, Hispanic/Latino Americans, American Indians, Asian Americans, Native Hawaiians or other Pacific Islanders are at a high risk for Type 2 diabetes and its complications (1; 2).

Complications Associated with Diabetes

Diabetes is not only an endocrine disease but also a vascular disease, as almost all diabetic complications leading to high mortality are associated with changes in vascular structure and function. Heart disease and stroke account for about 65 percent of mortality associated with diabetes (2). Adults with diabetes have a two to four-fold increased risk for these life-threatening conditions over non-diabetics (1). Hypertension is another wide-spread complication and about 73 percent of diabetics have blood

pressure over 130/80 mmHg or use prescription medications for hypertension (1; 2). Diabetes is also a leading cause of blindness associated with diabetic retinopathy, with nearly 14,000 new cases every year (1; 2). Kidney failure is a major pathological outcome due to diabetes, with a large majority of this population in end-stage renal disease, chronic dialysis or kidney transplant (1; 2). About 70 percent of people with diabetes also have mild to severe forms of nervous system damage, which is a major cause of lower-limb amputations (2). Periodontal disease, complications during pregnancy, biochemical imbalances such as ketoacidosis and non-ketotic coma and weakened defense mechanisms are other major causes of concern in patients with diabetes (2). Although diabetes is a major cause of mortality, the deaths are mostly due to its associated complications and not due to diabetes itself (1).

Metabolic Syndrome

Obesity, insulin resistance and hypertension often accompany diabetes resulting in a condition referred to as the metabolic syndrome or syndrome X (3; 4). The National Institutes of Health and American Heart Association formed a consortium that identified six major components of the metabolic syndrome related to cardiovascular disease – abdominal obesity, atherogenic dyslipidemia, elevated blood pressure, insulin resistance with or without glucose intolerance, a proinflammatory and a prothrombotic state (5). The multifaceted challenge that this syndrome presents therapeutically, is not well understood in large-part due to the lack of a relevant animal model available for pre-clinical research. While studies with obese Zucker rats or ob/ob mice provide important information about complications of obesity and pre-diabetes, relative

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contributions of the components of metabolic syndrome to diabetes-associated complications cannot be dissected. Several groups demonstrated that high fat diet-induced obesity alone can cause high blood pressure (6-8). In order to develop novel therapeutic targets and strategies, it is very important that the interaction of hyperglycemia and hyperlipidemia in diabetes are well defined. Thus, the Goto-Kakizaki rat model offers an excellent opportunity to study not only the individual roles of mild/moderate hyperglycemia (as seen in a majority of Type 2 diabetic patients) and hyperlipidemia in cardiovascular complications, but also the combined effects of the two.

Vascular Remodeling

Small artery structure is mainly comprised of three layers – intima, media and adventitia which together form the primary functional components of the vessel – the wall and the lumen. The active properties of the vessel are determined by individual vascular smooth muscle cell (VSMC) contraction, their number and arrangement. Thus the vascular structural properties such as wall and medial thickness (M) and lumen diameter (L) determine the physiologically relevant parameters such as M/L ratio at a particular intravascular pressure (9-11). The structure of resistance vessels is altered (remodeled) in hypertension, increased blood flow and changes in endothelial and hormonal components of the environment (9; 10).

Vascular remodeling can be of different kinds depending on etiology of the disease. In human essential hypertension, it is mostly eutrophic or inward remodeling, where there is a rearrangement of vascular wall components arrowed a narrowed

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lumen. Thus there is a decrease in total vessel diameter without a difference in wall area (9; 10). However, in other experimental models of hypertension, hypertrophic remodeling has been reported, with increased VSMC size (hypertrophy) and number (hyperplasia) which result in decreased lumen diameter and increased vessel wall cross-sectional area. Studies from animal models of hypercholesteremia have shown vascular remodeling characterized by neointimal growth that encroaches into the lumen (12). However, except for a few studies in experimental diabetes and obesity, most of the studies on small artery remodeling have been performed in various animal models of hypertension (13; 14). Thus, further studies involving hyperglycemia and hyperlipidemia in Type 2 diabetes are warranted.
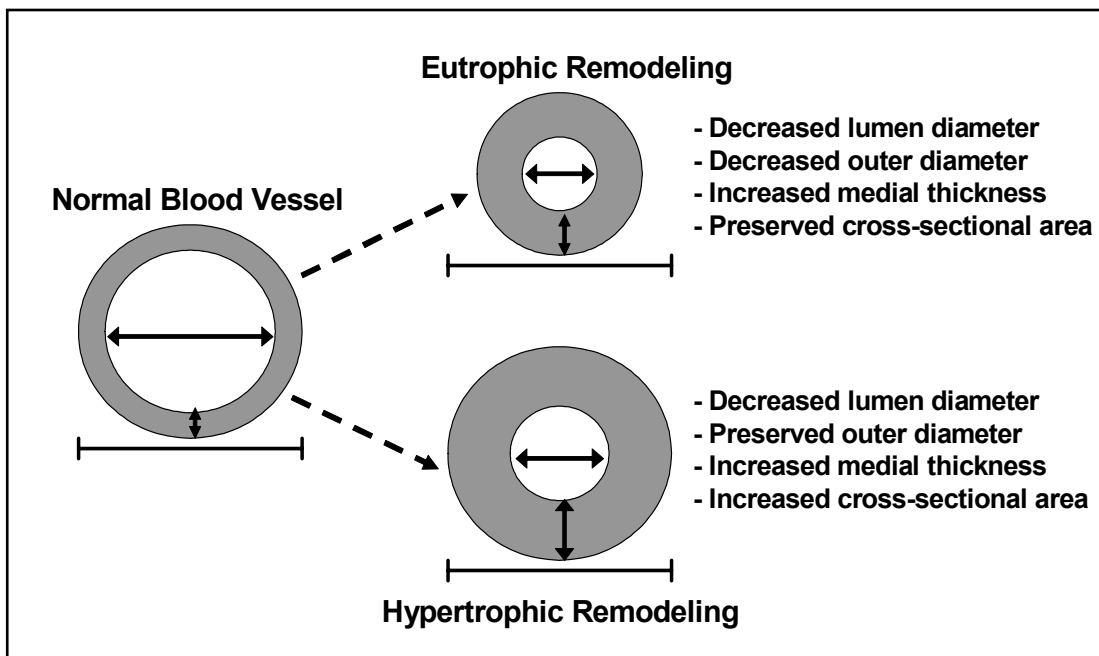
Figure 1.2. Types of Vascular Remodeling

Vascular Remodeling in Diabetes

Structural changes in the vasculature due to diabetes are characterized by vascular medial thickening and decreased diameter of the lumen, thus increasing the media-to-lumen ratio (M/L), an index of vascular remodeling (14-16). These effects were shown in the streptozotocin (STZ)-induced rat, a Type 1 diabetic model. Other studies have addressed the issue of microvessel remodeling in various hypertensive rat models (17-19). In leptin-receptor deficient mouse models, decreased vascular remodeling was reported following administration of a high-fat, atherogenic diet in a mouse model of carotid artery injury, suggesting a link between leptin and obesity in cardiovascular complications (12). Associated with structural changes of microvessels in hypertension, it has been reported that there are abnormalities in the expression and/or localization of extracellular matrix (ECM) proteins – mainly collagen and fibronectin (17; 18). Data from our laboratory suggests that there is significant medial thickening and collagen deposition in mesenteric as well as cerebral vessels of the Goto-Kakizaki rat, a mildly-hyperglycemic and non-obese model of spontaneous Type 2 diabetes (20; 21).

The matrix metalloproteinases (MMPs) are a family of zinc-dependant enzymes that are extensively involved in vascular remodeling mediated by ECM turnover (22; 23). The vascular MMPs comprise of the collagenases (MMP-1 and 13), gelatinase A (MMP-2) and gelatinase B (MMP-9) that breakdown gelatin/denatured collagen and type IV and type V collagens (basement membrane). In addition to degrading ECM proteins, MMPs 2 and 9 are also involved in proteolytically activating growth factors that stimulate vascular smooth muscle cell (VSMC) growth, migration and collagen synthesis (24). MMP-9 also plays a key role in accelerated atherosclerosis (25). Tissue inhibitors

of metalloproteases (TIMPs) are natural inhibitors that tightly regulate MMPs (22; 23). Thus, different species of MMPs or TIMPs maybe differentially regulated to stimulate collagen synthesis or VSMC growth/migration via MMP-2 and 9, as well as by affecting collagen degradation by decreasing MMP-1 or 13 activities.

Vascular Mechanical Properties

Mechanical properties of the vessel determine its compliance and adaptability to changes in pressure and shear stress. ECM components such as collagen and fibronectin are not only associated with structural changes manifested in microvessels, but are also linked with the mechanics of the vessel (17; 19). Distensibility and stiffness reflect the elasticity of the vessel and its ability to adapt to changes in hemodynamic stress. They are thus measures of vascular compliance (26). Myogenic tone is the intrinsic ability of the vascular smooth muscle cells to respond to changes in pressure and hemodynamic stress (26). Autoregulation is a phenomenon possessed to a greater degree in the cardiac, cerebral and renal circulations compared to others. This is a term used to describe the ability of the vasculature to control and limit perfusion to the organ they supply, and they do this by adjusting their functional lumen diameter (26). A large number of studies on the mechanical properties of resistance or cerebral vessels have been done in hypertensive animal models (17-19; 27-29). Ageing, even independent of diabetes is known to have deleterious effects on the microvasculature and in cardiovascular prognosis in the long run. It has been shown that there is outward hypertrophic remodeling and decreased compliance associated with ageing (30; 31). However, very little is known about the relative effect on diabetes, diabetic progression

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and the additional effect of diet-induced hyperlipidemia on the mechanical properties of the resistance vasculature. Data from our laboratory suggests that there is significant medial thickening and collagen deposition in mesenteric microvessels of the Goto-Kakizaki (GK) rat, a mildly-hyperglycemic and non-obese model of spontaneous Type 2 diabetes (21). Our group has shown increased gelatinolytic activity in the GK rat mediating vascular remodeling in the mesenteric circulation (21). Su et al. demonstrated that gelatinases, primarily MMP-9, to be involved in regulating microvessel tone (32). However, the correlation of these structural changes to the mechanical properties of the vessel, and the additional effect of a high-fat diet remain to be established. This was our rationale to examine the relative roles of hyperglycemia and diet-induced hyperlipidemia in causing changes in microvascular mechanical properties, and to study the involvement of MMPs in mediating these processes.

Vascular Function

Vascular dysfunction in microvessels, described by a hyperreactivity to vasoconstrictors or impaired relaxation to vasodilators, is associated with diabetes and hypertension (33). The vascular endothelium plays a prime role in regulating basal tone, permeability, coagulation and VSMC growth (34). It is an early target for attack in diabetes, and thus participates in the diabetic vascular disease process (35). The production of vasoactive factors by the endothelium is in a delicate balance. However, in diabetes, this ratio tends towards increased production of vasoconstrictors like endothelin-1 (ET-1) and decreased vasodilators like nitric oxide (NO) and prostaglandin I2 (36). ET-1 is also mitogenic in addition to being a potent vasoconstrictor, and has

been shown to mediate both vascular remodeling and dysfunction (37). Data from our

lab as well as reports from others suggest a hyperreactive response to vasoconstrictors

in mesenteric arteries of Goto-Kakizaki rats (38; 39). Studies involving fructose-fed rats

that were insulin resistant provide evidence for enhanced ET-1 activity and impairment

in relaxation mediated by specific pathways (40-42). Amiri et al. demonstrated in a

model of endothelium-specific ET-1 overexpression, that there is significant vascular

remodeling, increased vasoconstriction to ET-1 and impaired endothelial-mediated

relaxation in mesenteric microvessels (37). In the mesenteric arteries of the obese

Zucker rat, a leptin-deficient model of insulin resistance and metabolic syndrome,

impaired relaxation and reduced constriction is reported to exist, the net outcome being

vascular dysfunction (43). In a mouse model of diet-induced obesity, Molnar et al.

reported abnormal vascular function, however, not observing any vascular remodeling

(13).

Glycemic Control in Type 2 Diabetes

Hyperglycemia in cardiovascular disease is accompanied by a collection of risk

factors such as hypertension, dyslipidemia, endothelial dysfunction, hypercoagulability

and inflammation (44). It is fairly well accepted that glycemic control is the most

effective strategy in the prevention and treatment of diabetes and co-morbid obesity and

hypertension in cardiovascular disease (44; 45). Several randomized trials in Type 1

and 2 diabetes have been conducted, and meta-analyses have been performed (46;

47). The Diabetes Control and Complications Trial (DCCT) was one of the first studies

in effectively demonstrating the beneficial effects of strict glycemic control in a cohort

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with Type 1 diabetes on a decreased incidence of microvascular and neurological complications and also highlighted the importance of primary prevention (47). The UK Prospective Diabetes Study (UKPDS) was the largest clinical trial conducted in Type 2 diabetes, and established decreased incidence in both macro as well as microvascular events after treatment with various sulphonylureas, the biguanide metformin and/or insulin. Metformin lowered cardiovascular risk in obese patients with Type 2 diabetes, and prevented or delayed its onset in subjects with impaired glucose tolerance (5). While strong compelling evidence exists in favor of glycemic control in Type 1 diabetes, with only three major trials completed in Type 2 diabetes, the conclusions drawn are relatively modest in comparison (47). With strong pre-clinical data addressing key issues in Type 2 diabetes and with the completion of the large, on-going trial ACCORD (Action to Control Cardiovascular Risk in Diabetes), one would be able to better define the efficacy of tight glycemic control in improved prognosis in Type 2 diabetes (44; 47).

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CHAPTER 2

GLYCEMIC CONTROL PREVENTS MICROVASCULAR REMODELING AND IMPAIRED MECHANICS IN TYPE 2 DIABETES [1]

ABSTRACT

Medial thickening and vascular hypertrophy of resistance arteries can lead to worsened cardiovascular outcomes in diabetes. Most studies thus far have established the role of experimental Type 1 diabetes on vascular remodeling whereas, the effect of Type 2 diabetes, the more prevalent of the two is not well understood. Also, very little is known of whether and to what extent does reorganization of the extracellular matrix (ECM) affect vascular compliance and vasomotor tone. The onset and progression of these vascular changes in Type 2 diabetes and the effectiveness of glycemic control strategies in preventing or reversing these changes still remain unclear. Accordingly, this study assessed structural remodeling of mesenteric microvessels, vascular compliance and myogenic tone and the role of matrix metalloproteinases (MMP) in mediating these processes. Spontaneously diabetic, non-obese Goto-Kakizaki (GK) rats, a model for Type 2 diabetes and normoglycemic Wistar rats were used for the studies. A subset of GK rats were administered metformin (<300 mg/kg/day) to achieve euglycemia. The effect of diabetes progression on vascular remodeling was studied using GK rats at three different age groups. Metformin treatment normalized the increased media-to-lumen ratios (M/L), vessel stiffness and myogenic tone seen in diabetes. There was increased collagen turnover in diabetes paralleled by increased expression of collagenase MMP-13, while glycemic control attenuated the process. Diabetic progression was associated with increased vessel diameters and cross-sectional areas, suggesting adaptational outward remodeling. Early initiation of glycemic control could prove critical in preventing vascular remodeling and cardiovascular complications in diabetes.

INTRODUCTION

Cardiovascular disease is the leading cause of mortality in the United States and the presence of diabetes further increases the risk of morbidity and mortality by two to four-folds (1; 2). Nearly 21 million Americans are estimated to have diabetes, accounting to over 7% of the total population and 10% of all health care expenses. However, one-third of this population remains undiagnosed (1).

Cardiovascular homeostasis is governed in large part by the structural integrity of blood vessels. While larger vessels like the aorta provide capacitance, microvessels in the mesenteric circulation regulate total peripheral resistance. Alterations in vessel morphology due to restructuring of extracellular matrix (ECM) components is termed as vascular remodeling, and has been reported by several groups in animal models of hypertension and diabetes (14; 18; 27). In the streptozotocin (STZ) treated rat model of Type 1 diabetes, there is medial thickening and proliferation in the intima, thereby decreasing the functional media-to-lumen ratio (14-16; 48). The effect of Type 2 diabetes on vascular remodeling is not well understood, and these findings in Type 1 diabetes cannot be extrapolated due to extremely high blood glucose levels in STZ rats and the manual tissue fixation techniques employed thus far for morphometric evaluation of the vasculature.

Vascular compliance represented by the mechanical properties of the vessel, is an assessment of its ability to respond to physiological shear stress and pressure. In hypertension, it is fairly well established that there is increased myogenic tone and stiffness accompanied by decreased vessel distensibility, thus reducing overall compliance (17; 27; 28; 49). Age is an important determinant of vessel structure and

compliance especially in disease, and is critical in establishing the onset and progressive worsening of vascular mechanics. Laurant et al. reported age-related changes in mechanical properties of resistance vessels with differences in vascular compliance (31). In experimental salt-sensitive hypertension, resistance arteries have increased lumen diameters with age, accompanied by a decrease in distensibility (50). However, these studies were done either in control or hypertensive animals, and barring a few studies suggesting increased myogenic tone in ophthalmic arteries in Type 2 diabetes, not much is known about the impact of age on microvascular remodeling and compliance in relation to diabetic onset and progression (51; 52).

Matrix metalloproteinases or MMPs are a family of zinc-dependant enzymes that are widely implicated in ECM turnover (22; 23). Tight MMP regulation is critical to vascular structure, as they control the synthesis and degradation of ECM components, mainly collagen and fibronectin (53). Gelatinase MMPs 2 and 9 have been shown specifically to mediate eutrophic remodeling, neointimal growth and cell proliferation in rodent models of atherosclerosis (24; 54). MMP-13 is a collagenase that degrades fibrillar collagen (23). We previously demonstrated that vascular remodeling in diabetes in both the mesenteric and cerebral circulations is due to upregulation of the endothelin system, correlating with an increase in MMP activity and expression, and collagen turnover (20; 21). In addition, we reported differential gene expression of ECM components in micro as well as macro vessels in Type 2 diabetes (55). Thus, we proposed to investigate changes in microvascular structure and mechanics in Type 2 diabetes, the effect of diabetes progression and the employment of glycemic control in diabetes in relation to MMP function and extracellular matrix components (collagen).

Our hypothesis was that Type 2 diabetes will impair the structure and compliance of mesenteric arteries that will further worsen with disease progression, while glycemic control with metformin will prevent these pathological outcomes. These studies were done using control Wistar rats and non-obese and normotensive, Type 2 diabetic Goto Kakizaki rats at different ages to determine the role of diabetes progression on the vasculature.

METHODS

Animals

All experiments were performed on male control Wistar (Harlan, Indianapolis, IN) and diabetic GK (in-house bred, derived from the Tampa colony) rats. The animals were housed at the Medical College of Georgia animal care facility, approved by the American Association for Accreditation of Laboratory Animal Care. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC). Animals were fed standard rat chow and tap water ad-libitum until sacrifice, housed in individual cages and maintained on a 12-hour light-dark cycle. Metformin (<300 mg/kg/day) was administered in drinking water to a subset of GK rats. Treatment was initiated after the onset of diabetes in GK rats and continued until sacrifice at 18 weeks of age, accounting for at least 6 weeks of treatment. To study the effects of diabetes progression on vascular and metabolic parameters, two more subsets of GK rats were included –at 10 weeks and 24 weeks (referred to as diabetic 10 wk, 18 wk or 24 wk respectively). Blood glucose was measured twice weekly from the tail-vain using a commercial glucometer (Freestyle, Alameda, CA) for all the groups. Long term glucose

control was assessed from glycosylated hemoglobin values (A1C, Metrika Inc.,

Sunnyvale, CA). Blood pressure (Mean arterial pressure or MAP, and Pulse pressure,

mmHg) was measured twice a week from week 8 until sacrifice, by tail-cuff

plethysmography (Kent Scientific, Torrington, CT). This technique was validated on

telemetry transmitter-implanted animals to yield comparable results (56). At sacrifice,

the animals were anesthetized with sodium pentobarbital, ex-sanguinated via cardiac

puncture.

Surgical procedures

The mesenteric bed was harvested, immersed in ice-cold Kreb's-HEPES buffer

(calcium-free) and third-order mesenteric arteries were isolated in order to study their

structure and mechanical properties. The remaining arteries were processed for MMP

zymography and immunoblotting.

Plasma measurements

Plasma was obtained from heparinized blood and insulin, triglycerides, cholesterol and

endothelin-1 (ET-1) were measured. Plasma ET-1 and insulin were measured by

enzyme-linked immunosorbant assay kits (Alpco Diagnostics, Windham, NH). Plasma

triglycerides and cholesterol were measured using commercial kits (Wako Diagnostics,

Richmond, VA).

Vascular Structure

Vascular structure was assessed by morphometric analysis of Masson trichrome

stained mesenteric artery cross-sections. Third order mesenteric microvessels were

fixed in formalin using the quick-transfer freezing chamber (Living Systems

Instrumentation, Burlington, VT), wherein, they were maintained at a constant

intraluminal pressure in calcium-free Krebs-HEPES buffer (50 mmHg for 30 min) and frozen immediately. This procedure corrected for variations in vascular structure due to inconsistencies in manual perfusion. Images were captured and wall thickness, lumen and outer diameter measured from Masson stained cross-sections using SPOT software (Diagnostic Instruments, MI).

Morphometry was also studied using the pressurized arteriograph (Living Systems Instrumentation, Burlington, VT) under passive conditions using calcium-free Krebs-HEPES buffer. Medial thickness, lumen and outer diameters and vessel cross-sectional areas were measured over a range of pressures (0-120 mmHg at 20 mmHg increments) following earlier reports (27; 29). All data was plotted using GraphPad Prism version 4.03 (GraphPad Software Inc., San Diego, CA).

Collagen deposition patterns were evaluated in mesenteric artery cross-sections stained with picrosirius red captured under polarized light as previously described (57). Mature collagen stained red whereas newly formed collagen stained green. Collagen turnover was quantified using Metamorph software (Molecular Devices, CA) by measuring the intensities of green and red-stained regions. A green-to-red ratio would represent collagen turnover in these vessels. Collagens type 1 (Rabbit polyclonal antibody, Calbiochem, CA) levels were evaluated by slot-blot analysis as previously described (56). Protein levels were normalized using β-actin (A3854, Sigma Aldrich, Saint Louis, MO) as a loading control.

Vascular Mechanics

Third-order mesenteric artery segments were mounted on two glass cannulae (100-150μm diameter) and secured using 10-0 ophthalmic sutures in a small vessel

arteriograph (Living Systems Instrumentation, Burlington, VT). The distal cannula was closed off for a blind-sac experiment in conditions of zero-flow and the system was maintained at 5 mmHg (vessels collapse at 0 mmHg), placed under an inverted video microscope and equilibrated for 30 minutes. Medial thickness, lumen and outer diameters at different pressures ranging from 0(5)-120 mmHg were measured using a video dimension analyzer at 20 mmHg pressure increments. The time between each recording was between 4 to 5 minutes. This gave an indication of active vessel mechanics. Upon completion, the system was then equilibrated in Krebs-HEPES buffer free of calcium to study passive vessel mechanics and morphometry was recorded as previously described. Myogenic tone, stress, strain and stiffness (beta-coefficient) were calculated using earlier reports (29). Stress-strain curves were plotted using KaleidaGraph version 4.0 (Synergy software, Reading, PA). Stiffness coefficient β was obtained from the slope of the stress vs strain curve using the equation $y = ae^{\beta x}$.

Gelatin Zymography and Immunoblotting

Snap-frozen third-order mesenteric arteries were homogenized and gelatin zymography and immunoblotting were performed to determine activity and expression of MMPs. Expression of MMPs 2, 9 and 13 were determined. Snap-frozen mesenteric artery segments were placed in modified radioimmunoprecipitation assay (RIPA) buffer (50 mmol/l Tris-HCl, 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mmol/l NaCl, 1 mmol/l phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mmol/l sodium orthovanadate, and 1 mmol/l sodium fluoride) and sonicated at room temperature for 8- to 10-s bursts. Samples were placed on ice between sonications. Total protein was measured using the Bradford method (BioRad, Richmond, CA).

Vascular extracts (20 μg) were separated on 10% SDS gels and transferred to a nitrocellulose membrane in Tris-glycine transfer buffer supplemented with 20% methanol. The immunoblots were blocked for 1 h in 5% powdered goat milk diluted in 0.2 M Tris-base, 1.4 M NaCl, 0.1% Tween 20 and 0.02% NaN_3. The membranes were then incubated overnight with the primary antibodies (Research Diagnostics, Flanders, NJ). Bands were visualized using ChemiGlow (Alpha Innotech Corporation, San Leandro, CA). All densitometric measurements were normalized using an antibody against β-actin (Sigma Aldrich, Saint Louis, MO) as a loading control. Gelatin zymography was performed as previously described (20). Tissue inhibitors of metalloproteinase-2 levels (TIMP-2) were obtained using ELISA kits (Amersham Biosciences, Piscataway, NJ).

Statistical Analysis

One-way ANCOVA was performed between groups with body weight used as a covariate. A Tukey's mean separation test was used for significant results. SAS 9.1.3 was used for the analyses. A one-way ANOVA with repeated measures was done to determine group differences in morphometry over the range of pressures using the arteriograph, coupled with a post-hoc Tukey analysis. Graphpad Prism 5.0 was used for these analyses (Graphpad software, San Diego, CA). Significance was considered at $p < 0.05$. All results are reported as unadjusted mean ± SE.

RESULTS

Metabolic Parameters

Blood glucose was significantly elevated in diabetes, while metformin treatment normalized blood glucose (118 ± 6 mg/dl). Body weight was lower in diabetic rats compared to controls. Although the mean arterial pressure was comparatively lower in the diabetic 18wk group, pulse pressure was significantly higher.

Plasma cholesterol was mildly elevated in diabetes, whereas triglycerides were lower than in controls. Insulin levels in the plasma were increased in the diabetic 10wk group (2.6 ± 0.2 vs 1.5 ± 0.3 in Wistar; ng/ml) compared to controls, but in the 18 and 24 week-old diabetic counterparts the values were not different. The hyperinsulinemia seen early on in diabetes has been reported in GK rats (58), and proceeds to low plasma insulin levels with diabetes progression. However, studies from our lab show that these animals remained insulin resistant inspite of being insulinopenic and were glucose intolerant (20). Metformin treatment decreased plasma insulin levels in diabetes (0.7 ± 0.1 ng/ml). Blood glucose and hemoglobin A1C (Hb A1C) measures correlated tightly with changes in insulin levels. (Table 1).

Vascular Remodeling

Masson staining of mesenteric artery cross sections showed that diabetic rats had medial thickening compared to controls (red-stained regions) at 18 weeks of age, although total vessel size was not different. This denotes encroachment of vascular smooth muscle cells from the media into the lumen, thereby causing higher M/L ratios. However, this was not seen at 10 and 24 weeks in diabetic rats. Metformin treatment markedly attenuated vascular remodeling in diabetes, with M/L values comparable to controls (Figure 1).

Medial thickness, lumen and outer diameters of pressurized vessels were similar in control and diabetic rats at 18 weeks of age. However, with the progression of diabetes, there was an increase in lumen and outer diameters and a corresponding increase in the medial cross-sectional area (Figure 2). There was a change in body weight from 10 to 18 weeks in diabetes (Table 1), which could have resulted in larger vessel sizes. However, body weight was similar in diabetic 18 and 24wk groups, suggesting that the change in vessel dimensions was an adaptational effect.

Further, increased collagen staining was seen in the adventitia in diabetes, indicated by the blue regions (Figure 1). This correlates with patterns observed with picrosirius red staining (Figure 3). Diabetic animals have higher collagen turnover than controls, and metformin-induced euglycemia attenuates new synthesis of collagen in diabetes. Expression of type 1 collagen (Figure 4a and b) was increased in diabetes at 10 and 18 weeks of age.

Mechanical Properties

Myogenic tone was assessed at 60 mmHg from active and passive diameters of the vessel using the arteriograph. Hyperglycemia caused increased tone in the diabetic rats, whereas metformin treatment protected against increase in myogenic tone. Also, diabetes progression showed increased tone from 10 to 18 weeks, but not at 24 weeks (Figure 5a and b).

Vessel stiffness (β-coefficient) was calculated from the slopes of stress versus strain curves for each individual animal. Diabetic rats at 18 weeks had stiffer vessels than controls, but glycemic control with metformin prevented this effect. This increase in

stiffness was seen only at 18 weeks in the diabetic animals, and not at 10 and 24 weeks (Figure 5c and d).

MMPs in Vascular Remodeling

Gelatin zymography was done to determine enzymatic activity of MMPs 2 and 9. Representative images and densitometric analysis of lytic bands are shown in Figure 6a. Densitometric analysis revealed no significant differences between groups (Figure 6b). TIMP-2 levels were elevated in diabetes at 10 and 24 weeks compared to 18 weeks (Figure 6c). Expression of collagenase MMP-13 was elevated in 18 week-old diabetic rats compared to controls, but not in the other diabetic groups (Figure 4b), whereas there was no difference in MMP 2 and 9 protein levels between groups (data not shown).

DISCUSSION

The effects of Type 1 diabetes on vascular remodeling have been fairly well-studied (15; 16; 59; 60). However, the extent of structural remodeling of microvessels in Type 2 diabetes and the associated mechanics by which these occur are not clearly mapped out, partly because of the lack of a relevant animal model. Insulin resistance oftentimes clusters with Type 2 diabetes, either in the absence or presence of the other confounding factors such as dyslipidemia, obesity and hypertension that together form the metabolic syndrome or 'syndrome X'. The Goto-Kakizaki rat, which is Type 2 diabetic, non-obese and normotensive was thus a fitting animal model to address the effects of hyperglycemia alone in mediating vascular complications (61).

Vascular remodeling is an extensively researched concept in hypertension. Several groups have studied the structural and mechanical properties of either resistance mesenteric vessels or cerebral vessels in various animal models of hypertension, and have observed inward eutrophic remodeling in response to hemodynamic stress (17; 28; 29; 49; 50; 62; 63). In human essential hypertension, there is a rearrangement of vascular wall components arrowed a narrowed lumen. This is known as eutrophic remodeling. Thus there is a decrease in total vessel diameter without a difference in wall area (9; 10). However, in other experimental models of hypertension, hypertrophic remodeling has been reported, with increased VSMC size (hypertrophy) and number (hyperplasia) which result in decreased lumen diameter and increased vessel wall cross-sectional area (17; 18). Fukuda et al. established the presence of diabetes-induced vascular hypertrophy and remodeling in aortas of a drug-induced rat model of Type 1 diabetes (64). Similarly, the mesenteric microvasculature has also been shown to undergo hypertrophic remodeling due to increased medial mass and media-lumen ratios (16; 60). Laurant et al. reported outward hypertrophic remodeling in ageing control animals, wherein there was increased age-related vascular stiffness but no change in mesenteric arterial distensibility (31). However, there is still a paucity of data studying the effects of hyperglycemia, especially in Type 2 diabetes, and its role in vascular outcomes. Our study is one of the first to evaluate both structural and mechanical properties of microvessels in the mesenteric circulation in Type 2 diabetes. Moreover, using well-controlled procedures for fixing the microvessels, it was possible to accurately evaluate their morphology. Hypertrophic remodeling was observed in diabetes, which was effectively prevented by oral hypoglycemic drug, metformin. These

results tightly correlated with collagen turnover, which was increased in diabetes. In 24 week-old rats with comparitively advanced diabetes progression, media-to-lumen (M/L) ratios were not increased, but this was because of corresponding increases in vessel and lumen diameters, thus normalizing M/L ratios. While we observed differences in M/L with histochemical techniques, we could not reproduce this result using the arteriograph. One possible explanation is that in the relatively short time period (4 to 5 minutes) between morphometry recordings at various pressures in the arteriograph, the vessel could still be in a dynamic state resulting in overlap in vessel diameter. On the contrary, during tissue fixation with constant intraluminal pressure, the longer time frame (30 minutes) could allow the vessel to reach its threshold diameter and stabilize.

MMPs have been shown to play an important role in vascular dynamics by controlling the turnover of ECM components such as collagen and fibronectin. Our group recently established that differential MMP regulation resulted in altered ECM turnover patterns in the Type 2 diabetic GK rat (21). While in our current study we observed a difference in collagen turnover patterns which reflect ECM dynamics, there was no difference in MMP 2 and 9 activity or expression in this diabetic subset. The differences in MMP activity in this study in comparison to our earlier studies could be explained by number of variables such a source of the diabetic animals, their body weights, blood pressure and blood glucose profiles. It is well established that high glucose levels are associated with increased MMP activity and expression (65; 66). Indeed, the subset of diabetic animals used in our earlier studies had higher levels of blood glucose than those considered in the current study. Activity of the MMP system is also governed by the levels of TIMPs, which bind MMP enzymes in a 1:1 stoichiometry

(23). Tissue levels of TIMP-2, the natural inhibitor of MMP-2 were increased in diabetic

rats at 10 and 24 weeks, suggesting that the MMP system is in a dynamic state through

the course of diabetic progression. Expression of Type 1 collagen across the groups

followed a specific pattern that can be associated with the expression of the

collagenase MMP-13. In diabetes, there was increased expression of Type-1 collagen,

and a corresponding upregulation of MMP-13, probably to regulate collagen turnover. In

the diabetic 10wk group, type-1 collagen expression was increased, but not MMP-13.

The increase in MMP-13 expression in the diabetic 18wk group could be a

compensatory mechanism in order to restore normal collagen dynamics in diabetes.

Interestingly, in the diabetic 24wk group, the expression of both type-1 collagen and

MMP-13 are comparable to controls. Thus, there seems to be an association between

the collagen and collagenase expression patterns that needs to be further explored to

demonstrate a mechanistic approach to collagen regulation and vascular remodeling.

Mechanical properties of the vessel have been studied in great detail in

hypertensive animal models, where the systemic vasculature is generally believed to

possess altered myogenic tone and stiffness to counteract differences in hemodynamic

load (17; 27; 49). The same has been shown in the vasculature in the brain, where

there is increased stiffness and cerebral artery reactivity (26; 29). Su et al.

demonstrated a direct correlation between MMP-9 and resistance artery mechanics,

wherein the absence of the MMP-9 gene was associated with decreased myogenic tone

and improved endothelial-dependant vasodilation (32). Although it is known that MMP

enzymes are upregulated in diabetes, the effect of Type 2 diabetes-mediated changes

in vascular mechanics are not well understood. We did not find a difference in

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expression or activity of MMPs 2 and 9. However, we did observe an increase in myogenic tone as well as vessel stiffness, accompanied by an increase in collagen deposition and turnover, thus suggesting an active interaction between vascular structure and mechanics. We have established in an earlier study that in this model of Type 2 diabetes there is an increased nitrotyrosine level in mesenteric arteries (56). It is thus possible that there is an association between increased oxidant stress and type-1 collagen levels in mediating vessel stiffness and impaired mechanical properties. Further, the increase in lumen and outer vessel diameters that we see in the Diabetic 24wk group could be linked to the restoration of vascular mechanics that were impaired in the Diabetic 18wk subset. Future studies in this area will confirm the role of ageing in mediating vessel structure and mechanical properties.

The use of glycemic control to prevent or reverse vascular outcomes manifested by diabetes, although seemingly rational, has not yielded promising results in clinical trials (45; 67). A small clinical study focusing on inflammatory mediators in diabetes established that glycemic control with PPAR-γ agonist rosiglitazone increased adiponectin and decreased resistin levels, thus improving insulin sensitivity (68). However, beyond the changes in plasma biomarkers, its effect in mediating vascular compliance and function were not studied. Metformin, also an insulin sensitizer has been shown to improve vascular function in insulin resistant rats. Katakam et al. reported that it works independently of its metabolic effects in restoring impaired endothelial-dependant vasorelaxation in fructose-fed insulin-resistant rats (69). More recently, Rosen et al. demonstrated that the generation of reactive oxygen species *in vivo* played an important role in the onset of diabetes and the development of vascular

dysfunction in the Goto-Kakizaki rat model of Type 2 diabetes. The use of metformin delayed the onset and progression of diabetes and improved vascular function by normalizing impaired relaxation to acetylcholine and preventing hyperreactivity to phenylephrine (70). Rodriguez et al. studied the effects of pioglitazone in both the cardiac and renal circulations, and further underscored the beneficial effects of glycemic control mediated via anti-inflammatory mechanisms in a diet-induced rodent model of obesity and Type 2 diabetes (71; 72). In our study, we established that glycemic control with metformin was successful in preventing impairment in vascular structure, mechanics and compliance manifested by Type 2 diabetes. This is the first study to report the vascular effects of hyperglycemia and diabetes progression in a spontaneously developing, lean model of Type 2 diabetes. Further studies using clinically relevant animal models are required to justify and endorse the employment of glycemic control as a therapeutic as well as a primary prevention strategy in preventing vascular complications.

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Table 2.1. Metabolic parameters in Type 2 diabetes.

	Control	Diabetic	Diabetic-Euglycemic	Diabetic 10 wk	Diabetic 24 wk
Weight	481.9 ± 14.9	349.6 ±11.3 *	314.3 ± 7.2 *	263.0 ± 4.7*,**	389.0 ± 6.2 *
Glucose	99.1 ± 9.4	158.7 ± 12.9 *	118.0 ± 6.4*,**	190.1 ± 18.4 *	152.8 ± 16 *
Hb A1C	5.43 ± 0.06	7.61 ± 0.57 *	5.56 ± 0.07**	8.05 ± 0.76 *	na
Pulse pressure	45.8 ± 1.3	50.3 ± 1.1 *	48.6 ± 0.7	49.3 ± 0.8	na
MAP	117.6 ± 3.1	108.4 ± 1.5 *	118.0 ± 6.4	116.4 ± 2.8	119.5 ± 2.3
Insulin	1.5 ± 0.3	1.2 ± 0.2	0.7 ± 0.1 *	2.6 ± 0.2*,**	1.6 ± 0.3
Cholesterol	70.5 ± 4.6	88.6 ± 3.5 *	89.4 ± 3.5 *	100.5 ± 6.0 *	73.5 ± 14.7
Triglycerides	63.1 ± 2.6	37.8 ± 0.5 *	46.7 ± 5.6	43.8 ± 5.5 *	52.9 ± 2.8 *

*p<0.05 vs Control, **p<0.05 vs Diabetic (Mean ± SE; n=4-10/group; Control, Diabetic and Diabetic-Euglycemic groups were studied at 18 weeks.)

(Units: Weight – g, Glucose – mg/dl, Hb A1C - %, Mean arterial pressure (MAP), Pulse pressure – mmHg, Insulin – µg/l, Cholesterol, Triglycerides – mg/dl).

Figure 2.1

a)

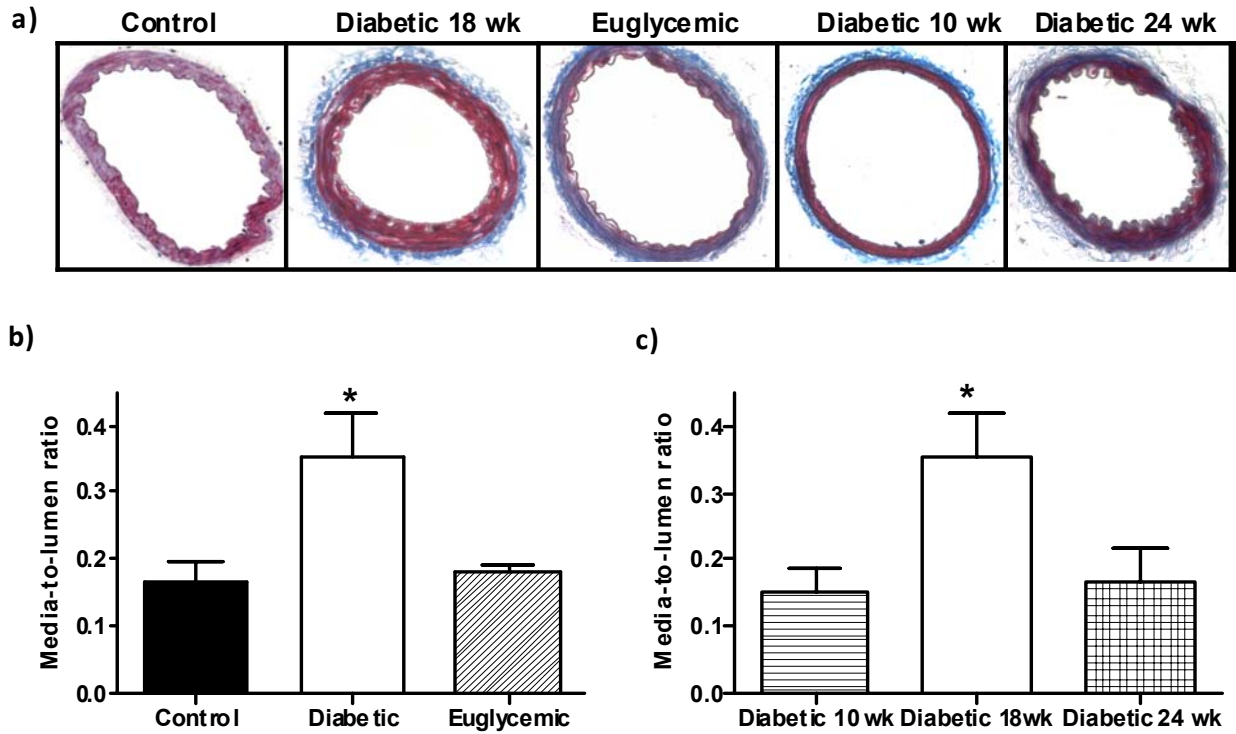| Control | Diabetic 18 wk | Euglycemic | Diabetic 10 wk | Diabetic 24 wk |

b)

c)

FIGURE 2.1. Vascular remodeling in Type 2 diabetes. Formalin-fixed mesenteric

artery cross-sections were analyzed for morphological changes and collagen deposition

by Masson staining. Diabetes induced nearly a two-fold increase in M/L ratio and

glycemic control with metformin prevented this increase. There were no differences in

M/L ratios at 10 and 24 weeks compared to control. Representative images are shown

in Panel a) and combined analysis is given in Panel b). *$p<0.05$ vs control, n=3-5/group.
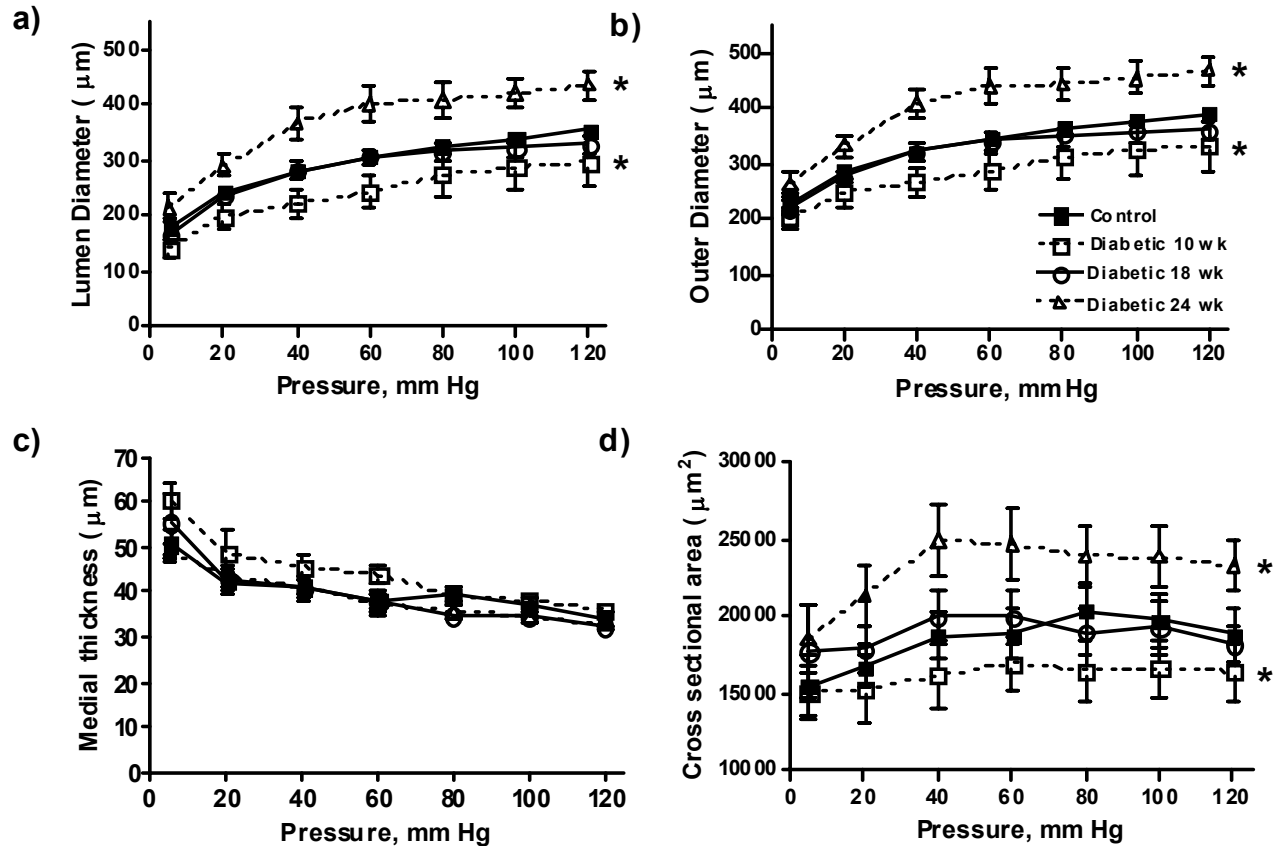
Figure 2.2

FIGURE 2.2. **Vascular remodeling in Type 2 diabetes.** Mesenteric artery

morphometry was evaluated using the pressurized arteriograph to validate the

histochemical approach. Inner and outer vessel diameters (**a** and **b**), medial thickness

(**c**) and total cross sectional area (**d**) were calculated over various pressures ranging

from 0 to 120 mmHg. There were no differences in pressurized vessels of diabetic

animals at 18 weeks compared to controls. However diabetes progression from 10 to 24

weeks lead to increased lumen and outer diameters and thereby increasing cross-

sectional areas of microvessels in diabetes, suggestive of outward remodeling. *$p<0.05$

vs control, n=6-8/group (except in Diabetic 24 wk group where n=4).

Figure 2.3
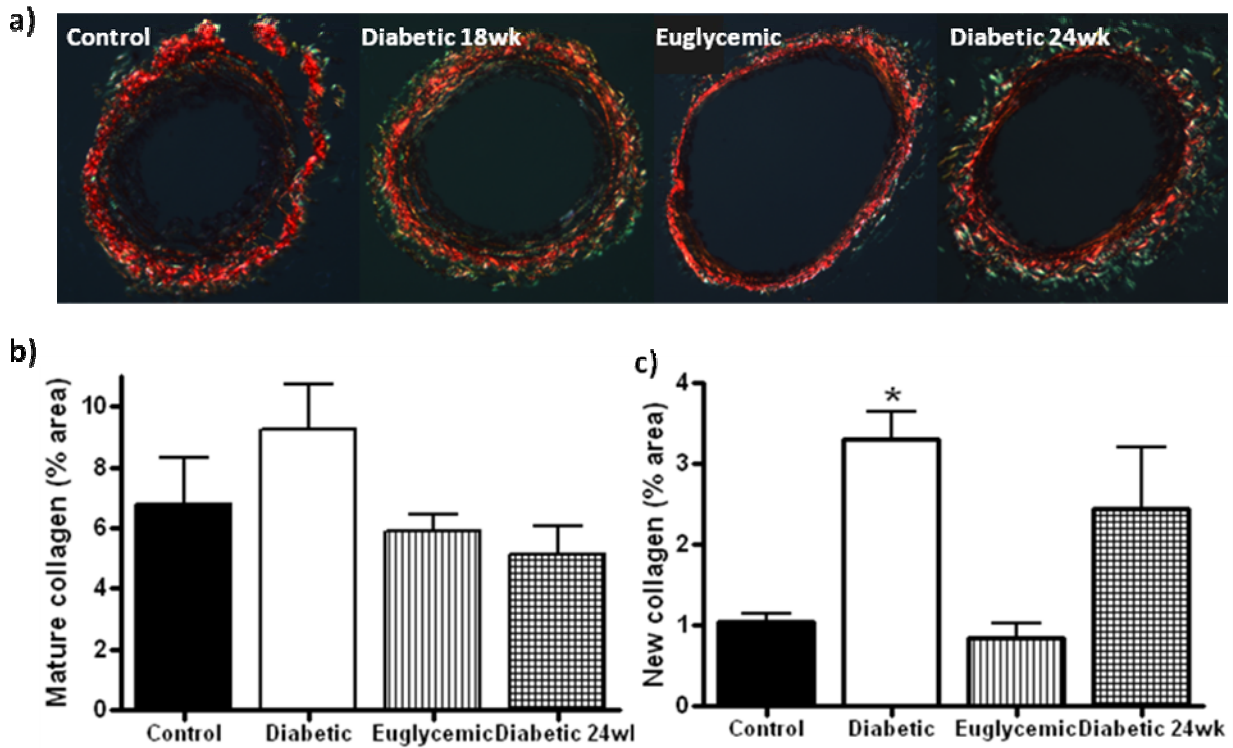
FIGURE 2.3. **Collagen turnover in Type 2 diabetes.** Collagen deposition patterns were observed from picrosirius red stained cross-sections, viewed under polarized light. There was increased collagen turnover in diabetes compared to control (increased green/red ratio in staining) and metformin treatment prevented differential collagen turnover. Representative images are shown in panel **a)** and combined analysis is given in panel **b)**. *p<0.05 vs control, n=3-5/group.

Figure 2.4
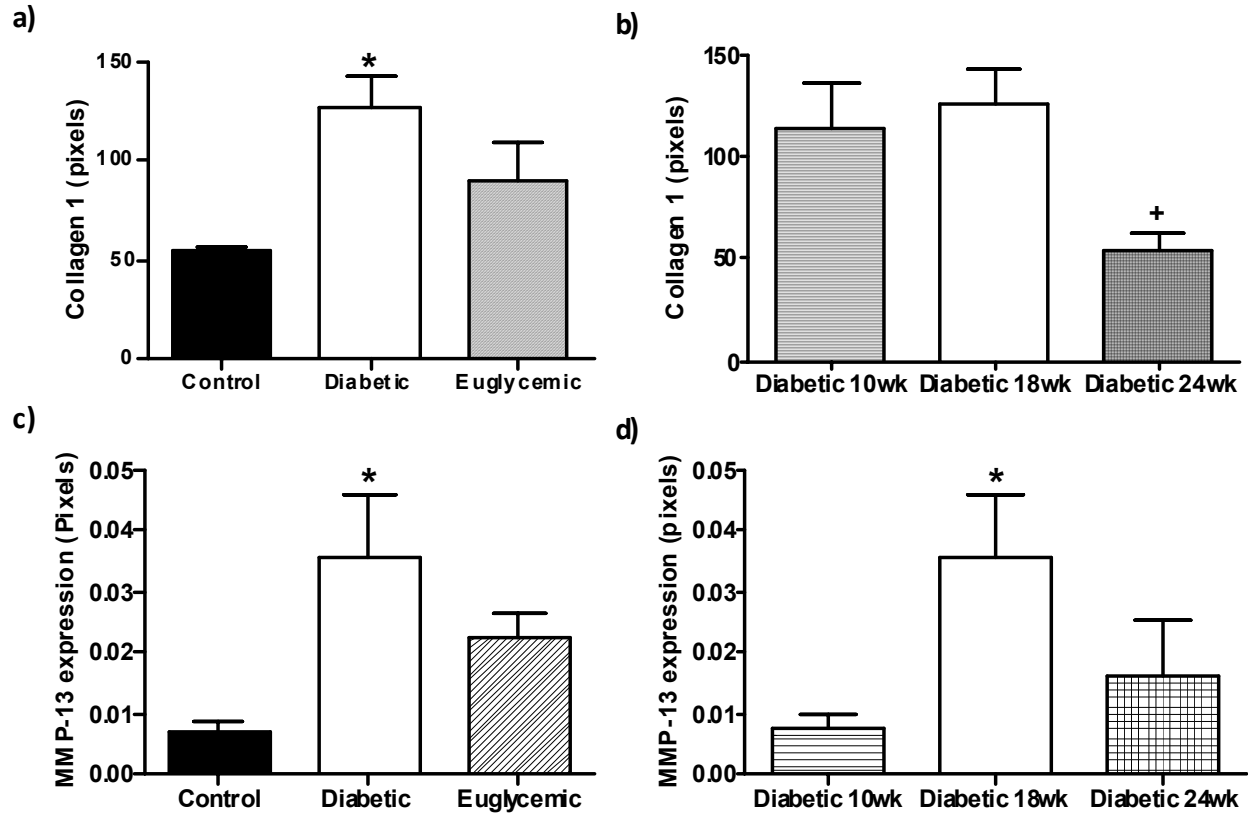
FIGURE 2.4. Type 1 collagen and collagenase MMP-13 expression. Expression of type 1 collagen and collagenase, MMP-13 were determined by immunoblotting. Densitometric analysis showed increased expression of type 1 collagen in diabetic animals at 10 and 18 weeks seen in Panel **a)**. MMP-13 expression was increased in diabetic rats at 18 weeks (Panel **b**). Densitometry values reported have been normalized to β-actin levels in all samples to account for differences in loading. *p<0.05 vs control;n=4-6/group.

Figure 2.5
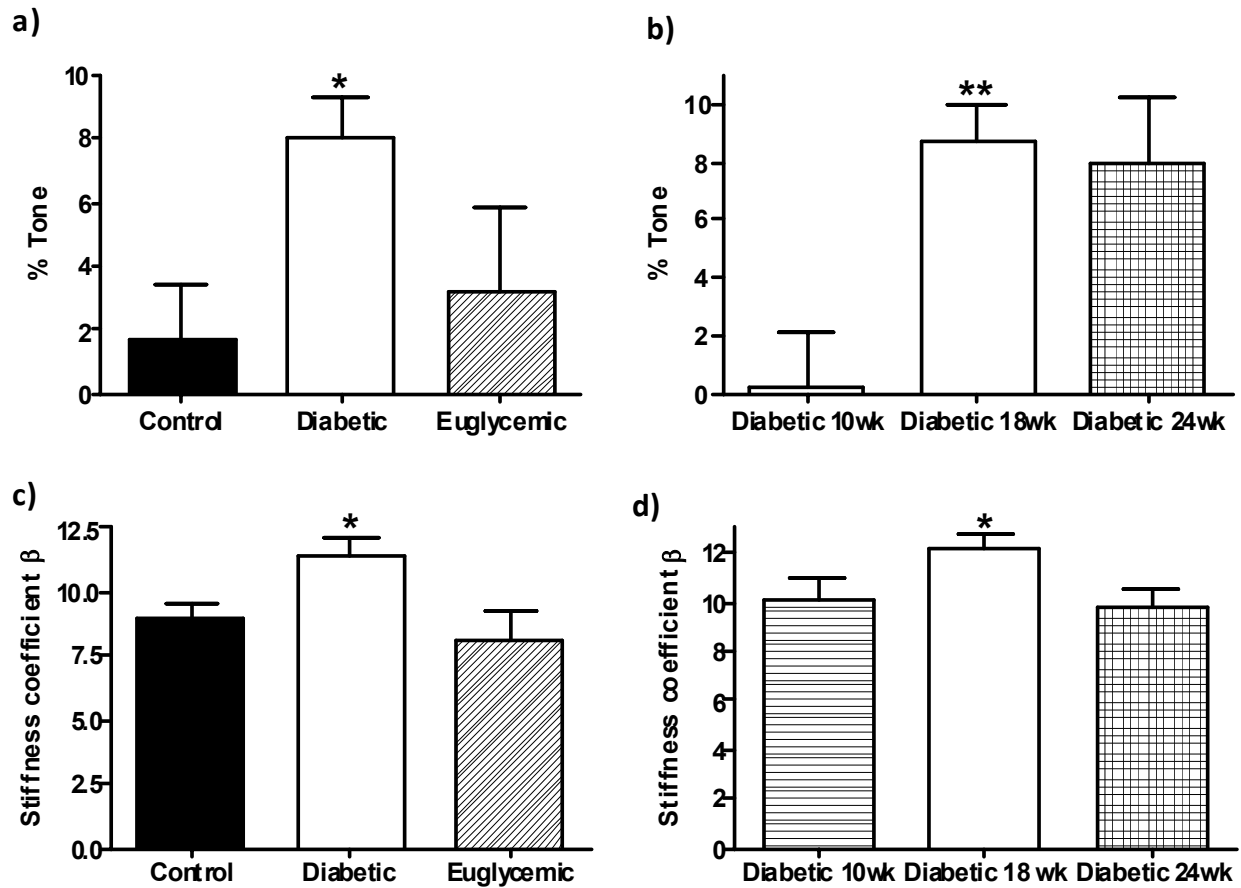
FIGURE 2.5. Mechanical properties in Type 2 diabetes. a) Myogenic tone was assessed with the arteriograph at 60 mmHg. Hyperglycemia increased microvascular tone in diabetes, thus impairing vessel compliance; glycemic control with metformin prevented this phenomenon. Increased vessel tone was seen only at 18 weeks in diabetes, but not at 10 and 24 weeks. b) Coefficient of stiffness or β-coefficient was increased in diabetes but not with metformin treatment. As in myogenic tone, increased stiffness was seen only at 18 weeks in diabetes and not at 10 or 24 weeks compared to control. $p<0.05$ vs control, n=6-8/group (except in Diabetic 24 wk group where n=4).

Figure 2.6
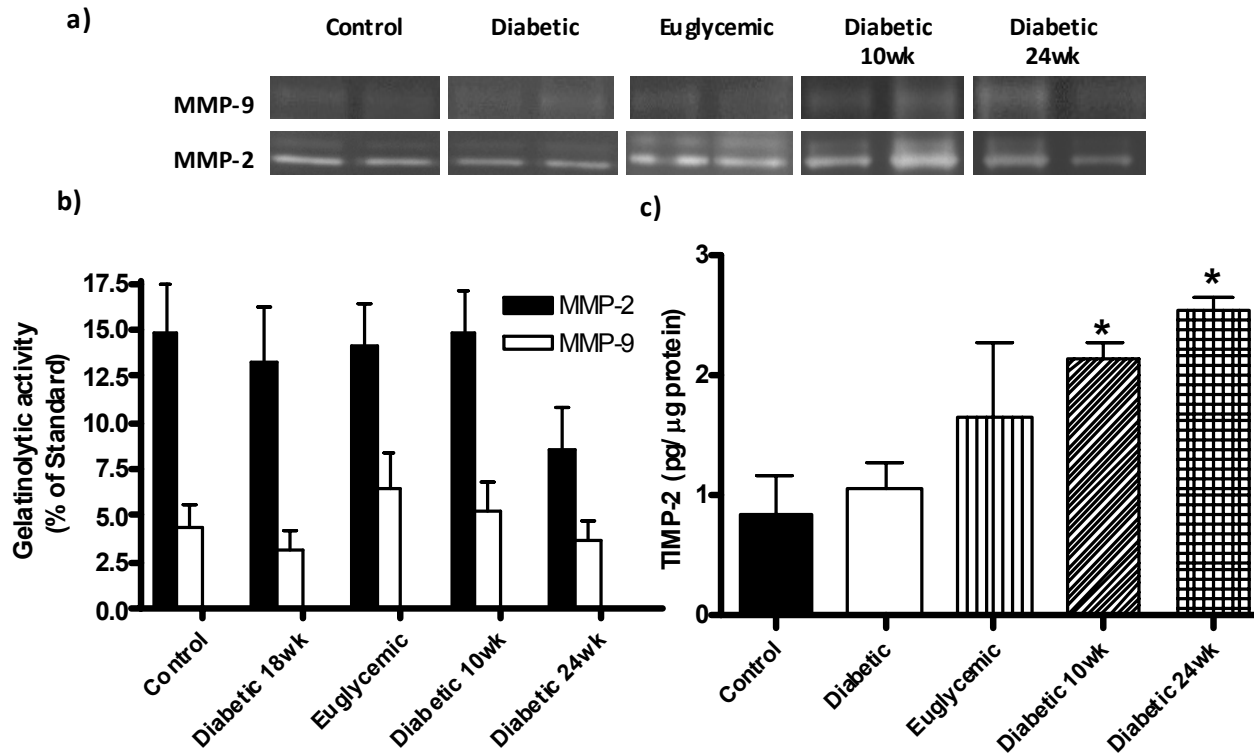
FIGURE 2.6. **Gelatinolytic activity in Type 2 diabetes.** Enzymatic activity of MMPs 2 and 9 was analyzed by gelatin zymography. Representative images of MMP-2 lytic bands are shown in panel **a**. Densitometric analysis of lytic bands for MMP-2 and 9 respectively (panels **b** and **c**) revealed no differences between groups. Densitometry values reported have been normalized to β-actin levels in all samples to account for differences in loading. TIMP-2 levels were increased in diabetic rats at 10 and 24 weeks but not at 18 weeks, suggesting a dynamic state of the MMP system. *p<0.05 vs control;n=4-6/group.

CHAPTER 3

DIET-INDUCED HYPERLIPIDEMIA IN TYPE 2 DIABETES – ROLE IN VASCULAR REMODELING AND COMPLIANCE [1]

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To be submitted to *Diabetes.*

ABSTRACT

Obesity, insulin resistance and hypertension often cluster with diabetes and form the metabolic syndrome, posing a major challenge on the vasculature. However, the relative as well as combined roles of diabetes and diet-induced hyperlipidemia in mediating changes in microvascular structure and mechanics are poorly understood. Control Wistar rats and spontaneously diabetic Goto-Kakizaki (GK) rats fed either a regular or high-fat diet were used. Pressurized third order mesenteric arteries were evaluated for mechanical properties, and morphometry was examined in perfusion-fixed vessels. Media to lumen ratios and myogenic tone were increased in diabetes, but this was absent with high-fat diet feeding. Gelatinolytic activity of MMPs was not different between groups, although TIMP-2 levels were increased in combined hyperglycemia and hyperlipidemia and MMP-2 protein levels were elevated with the high-fat diet. Vessel stiffness was increased in both the diabetic subsets, paralleled by an increase in MMP-13 expression. Diabetes decreased compliance, increased myogenic tone and caused microvascular remodeling. Addition of a high-fat diet altered vascular mechanics and increased cardiovascular risk factors in diabetes, which may contribute to further vascular complications. Thus, both hyperglycemia and hyperlipidemia need to be targeted for effective prevention and treatment of diabetic vascular disease.

INTRODUCTION

It has been estimated that over 18 million Americans are affected by Type 2 diabetes. Cardiovascular disease is the leading cause of diabetes-related death, with a two to four-fold increased risk in morbidity and mortality over non-diabetics. Obesity, insulin resistance and hypertension often cluster along with diabetes resulting in a condition referred to as the metabolic syndrome or syndrome X, thus imposing an enormous task from a therapeutic standpoint (3; 4). The multifaceted challenge that this syndrome presents is not well understood in large-part due to the lack of a relevant animal model available for pre-clinical research. While studies with obese Zucker rats (73-75) and ob/ob mice (12) provide important evidence about complications of obesity, insulin resistance, hypertension and pre-diabetes in the systemic microvasculature, the relative contributions of the individual components of metabolic syndrome to diabetes-associated complications cannot be dissected. It has been demonstrated that high fat diet-induced obesity alone can cause high blood pressure (6; 76; 77). In order to define specific targets and develop therapeutic strategies to treat vascular complications, it is very important that the interaction of hyperglycemia and hyperlipidemia in diabetes are well defined. Thus, the Goto-Kakizaki rat model offers an excellent opportunity not only to study the individual role of mild to moderate hyperglycemia (which is the case in a vast majority of Type 2 diabetic patients), but also its effects in combination with diet-induced hyperlipidemia in mediating vascular complications.

Structural changes in the vasculature due to diabetes are characterized by vascular medial thickening and decreased diameter of the lumen, thus increasing the media-to-lumen ratio (M/L), an index of vascular remodeling (14-16). These effects were

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shown in the streptozotocin (STZ)-induced rat, a Type 1 diabetic model. Other studies have addressed the issue of microvessel remodeling in various forms of hypertension (17; 18). In leptin-receptor deficient mouse models, decreased vascular remodeling was reported following administration of a high-fat, atherogenic diet, suggesting a link between leptin and obesity in cardiovascular complications (78).

The matrix metalloproteinases (MMPs) are a family of zinc-dependant enzymes that are extensively involved in vascular remodeling mediated by ECM turnover (22). Gelatinase A (MMP-2) and gelatinase B (MMP-9) breakdown gelatin/denatured collagen and type IV collagen. MMPs 2 and 9 (gelatinases) and are also involved in proteolytically activating growth factors that stimulate vascular smooth muscle cell (VSMC) growth, migration and collagen synthesis (24). MMP-9 also plays a key role in accelerated atherosclerosis (25). Collagenase MMP-13 degrades fibrillar collagen (23). Tissue inhibitors of metalloproteases (TIMPs) are natural inhibitors that tightly regulate MMPs (23). Studies from our lab have shown that in Type 2 diabetes, there is an upregulation of the MMP system in both the systemic as well as the cerebral circulations, contributing to vascular remodeling (20; 21). However, the role of MMPs in mediating vascular remodeling in combined hyperglycemia and hyperlipidemia is not well understood.

Mechanical properties of the vessel dictate its compliance and adaptability to changes in pressure and shear stress. Associated with structural changes of microvessels in hypertension, it has been reported that there are abnormalities in the expression and/or localization of extracellular matrix (ECM) proteins – mainly collagen and fibronectin (17; 18). Vessel stiffness governs distensibility and compliance in

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response to a dynamic environment (19). Myogenic tone is the intrinsic ability of the vascular smooth muscle cells to respond to changes in pressure and hemodynamic stress (79). However, very little is known about the effect on diabetes and diet-induced hyperlipidemia on the mechanical properties of the resistance vasculature. Data from our laboratory suggests that there is significant medial thickening and collagen deposition in mesenteric microvessels of the Goto-Kakizaki (GK) rat, a mildly-hyperglycemic and non-obese model of spontaneous Type 2 diabetes (21). However, the correlation of these structural changes to the mechanical properties of the vessel, and the additional effect of a high-fat diet remain to be established. Accordingly, this study sought to examine the relative roles of hyperglycemia and diet-induced hyperlipidemia in microvascular remodeling and mechanics, and the involvement of MMPs in mediating these processes.

METHODS

Animals and treatment

All experiments were performed on male control Wistar (Harlan, Indianapolis, IN) and diabetic GK (in-house bred, derived from the Tampa colony) rats. The animals were housed at the Medical College of Georgia animal care facility, approved by the American Association for Accreditation of Laboratory Animal Care. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC). Four experimental groups were included in study – a control Wistar and diabetic GK group and a control and diabetic group administered the same quantity of a high-fat diet through the study period. The high-fat diet contained 36% fat (15.2% saturated and

20.8% unsaturated), 35% carbohydrate and 0.4% salt (Cat.# F2685, Bio Serv, NJ) (7; 8) and control diet that had 4.4% fat (2.5% saturated and 1.9% saturated) 46.6% carbohydrate, and 0.3% salt and was given from week 11 through week 18. All animals were placed in metabolic cages for a 24-hour period at the beginning, middle and end of the treatment phase and food and water intake were monitored. Blood glucose was measured twice weekly from the tail-vein using a commercial glucometer (Freestyle, Alameda, CA). After week 18, animals were sacrificed and blood was collected in heparinized vials. Long term glucose control was assessed from glycosylated hemoglobin values (A1C, Metrika Inc., Sunnyvale, CA). Blood pressure (MAP, mmHg) was measured using the tail-cuff method (Kent Scientific, Torrington, CT). MAP was recorded from week 8 through week 18, twice a week. This technique was validated on telemetry transmitter-implanted animals to yield comparable results (56).

Surgical procedures

Animals were anesthetized with sodium pentobarbital and ex-sanguinated via cardiac puncture. The mesenteric bed was harvested, immersed in ice-cold Krebs-HEPES buffer (calcium-free) and third-order mesenteric arteries were isolated for vascular structure and mechanics studies, while the remaining was snap-frozen for immunoblotting and gelatin zymography. Adipose tissue from the visceral and epididymal depots was collected and weighed.

Plasma measurements

Plasma endothelin-1 (ET-1) and insulin were measured by enzyme-linked immunosorbant assay kits (Alpco diagnostics, Windham, NH). Plasma triglycerides and cholesterol were measured using commercial kits (Wako diagnostics, Richmond, VA).

Vascular Structure

Vascular structure was assessed by morphometric analysis of Masson trichrome stained mesenteric artery cross-sections. Third order mesenteric microvessels were fixed in formalin using the quick-transfer freezing chamber (Living Systems Instrumentation, Burlington, VT), wherein, they were perfused at a constant rate (50 mmHg for 30 min) and fixed in formalin simultaneously. This procedure controlled for variability with manual perfusion-mediated changes in vascular structure. Images were captured and wall thickness, lumen and outer diameter measured from Masson stained cross-sections using SPOT software (Diagnostic Instruments, MI).

Collagen turnover patterns were evaluated in mesenteric artery cross-sections stained with picrosirius red captured under polarized light as previously described (57). Mature collagen stained red or orange whereas newly formed collagen stained green or yellow. Collagen turnover was quantified using Metamorph software (Molecular Devices, CA) by measuring the intensities of green and red-stained regions. Thus, a green-to-red ratio was calculated to represent collagen turnover in these vessels. Collagens type 1 (Rabbit polyclonal antibody, Calbiochem, CA) and type 3 (Rabbit polyclonal antibody, Abcam, Cambridge, MA) and fibronectin (Mouse monoclonal antibody, Chemicon, Temecula, CA) levels were evaluated by slot-blot analysis as previously described (21). Protein levels were normalized using β-actin (A3854, Sigma Aldrich, Saint Louis, MO) as a loading control.

Morphometry was also studied using the pressurized arteriograph (Living Systems Instrumentation, Burlington, VT) under passive conditions. Medial thickness, lumen and outer diameters and vessel cross-sectional areas were measured over a

range of pressures (0-120 mmHg at 20 mmHg increments). In addition, remodeling and growth indices were calculated at 60 mmHg as follows:

Wall Thickness (WT, µm) = Outer Diameter – Lumen Diameter (ie, OD – LD)

Media/Lumen Ratio = WT/LD

Cross-sectional area ($µm^2$) = Outer vessel area – Lumen area

These formulae were adapted using an earlier report in hypertension (27). All graphs were plotted using GraphPad Prism version 4.03 (GraphPad Software Inc., San Diego, CA).

Vascular Mechanics

Third-order mesenteric artery segments were mounted on two glass cannulae (100-150µm diameter) and secured using 10-0 ophthalmic sutures in a small vessel arteriograph (Living Systems Instrumentation, Burlington, VT). The distal cannula was closed off for a blind-sac experiment in conditions of zero-flow and the system was maintained at 5 mmHg (vessels collapse at 0 mmHg) equilibrated in Krebs-HEPES buffer. The vessel was placed under an inverted video microscope and equilibrated for 30 minutes. Medial thickness, lumen and outer diameters were measured using a video dimension analyzer at different pressures ranging from 0(5)-120 mmHg at 20 mmHg pressure increments. The time between each reading was between 4 to 5 minutes. This gave an indication of active vessel mechanics. Upon completion, the system was then equilibrated in calcium-free Krebs-HEPES buffer to study passive vessel mechanics and morphometry was recorded as previously described. Myogenic tone, stress, strain and stiffness (beta-coefficient) were calculated as follows:

Myogenic tone (% tone) = (1 – (active OD/passive OD)) x 100

Circumferential Wall Stress = (Intraluminal Pressure x LD)/(2 x WT)

Circumferential Wall Strain = (LD – LD at 0 mmHg)/(LD at 0 mmHg)

Stress-strain curves were plotted using KaleidaGraph version 4.0 (Synergy software, Reading, PA). Stiffness coefficient β was obtained from the slope of the stress vs strain curve using the equation $y = ae^{\beta x}$.

MMP Activity and Expression

Snap-frozen third-order mesenteric arteries were homogenized and gelatin zymography and immunoblotting were performed to determine activity and expression of MMPs 2, 9 and 13 as previously described (20). All densitometric measurements were normalized to β-actin (A3854, Sigma Aldrich, Saint Louis, MO) used as a loading control. Tissue inhibitors of metalloprotease-2 levels (TIMP-2) were obtained using ELISA kits (Amersham Biosciences, Piscataway, NJ).

Statistical Analysis

Two-way ANOVA was performed comparing disease (control vs. diabetes) and diet (normal vs. high-fat diet), and a post-hoc Bonferroni analysis was done to determine significance between groups. Graphpad Prism 5.0 was used for all analyses (Graphpad software, San Diego, CA). Significance was considered at $p < 0.05$. All results are reported as unadjusted mean ± SE.

RESULTS

Animal metabolics

Weight gain was not different between diabetic and control animals on a normal diet. High-fat feeding caused accelerated weight gain in both strains of animals. The

mild degree of hyperglycemia seen in diabetic animals was markedly elevated with a high-fat diet. These values correlated with the long term glucose control depicted by hemoglobin A1C levels that were increased in diabetic rats and even further in those on a high-fat diet. Mean arterial pressure was comparatively lower in both the diabetic subsets than in control animals.

As observed in our previous studies, diabetes did not elevate plasma cholesterol levels in the GK rats; however, the high-fat diet raised cholesterol levels only in diabetic animals and not in the normoglycemic controls. The lower basal triglyceride level in this diabetic rat model was elevated to control values with high-fat feeding. Adiposity was increased in both control and diabetic groups on a high-fat diet. Hyperinsulinemia was seen in combined hyperglycemia and hyperlipidemia alone, and not in the other subsets. Endothelin-1 (ET-1) levels in the plasma were higher in diabetic rats, and further increased with the high-fat diet. (Table 3.1).

Vascular Structure

Medial thickening and narrowed lumens were observed in diabetes, thus increasing the overall media-to-lumen ratio (M/L). Diet-induced hyperlipidemia did not cause furthere elevation in M/L in diabetic animals (Figure 3.1). Vessel morphometry (outer and inner vessel diameters, medial thickness and vessel cross sectional area) was recorded under passive conditions using the pressurized arteriograph, and was similar between groups over pressures ranging from 0 to120 mmHg (data not shown).

Collagen turnover was quantified from picrosirius red stained images captured under polarized light. Mature collagen that appeared red or orange was quantified separately from newly formed collagen that was green or yellow colored, and a ratio of

new to old collagen was calculated to denote collagen turnover. Diabetic animals demonstrated increased collagen dynamics compared to controls, wherein the staining was mainly localized to the adventitia. High-fat feeding did not further alter collagen regulation in both control and diabetic animals (Figure 3.2a and 3.2b). However, type 1 collagen expression was increased in diabetic animals on a high-fat diet, although there was a strong trend for increased levels in both diabetes and hyperlipidemia taken alone as well (Figure 3.2c) and type 3 collagen was unchanged (data not shown).

Matrix metalloproteinase activity and expression

Gelatinolytic activity of MMPs 2 and 9 were quantified from zymogram gels. There was no difference in enzyme activity between the groups. MMP protein levels were quantified by western blotting (Figures 3.3a and b). TIMP-2 levels were assessed by ELISA and found to be increased in combined hyperglycemia and hyperlipidemia alone (Figure 3.3c). There was an increase in MMP-2 expression with high-fat feeding (Figure 3.4a), although MMP-9 protein levels were similar (data not shown). MMP-13 expression was increased in diabetic animals on either normal or high-fat diet (Figure 3.4b).

Vascular Mechanics

Coefficient of stiffness or the beta-coefficient which is indicative of vessel compliance was elevated in both the diabetic groups compared to the control groups. There was no exacerbated effect upon addition of a high-fat diet to diabetes. (Figure 3.5a). Myogenic tone was calculated from the active and passive morphometry of the vessel pressurized at 60 mmHg. Diabetic rats exhibited increased tone compared to

control rats. However, in diabetic rats fed the high-fat diet, myogenic tone was normalized to control values (Figure 3.5b).

DISCUSSION

Metabolic syndrome presents as a complex combination of insulin resistance, obesity, dyslipidemia and hypertension, posing an immense therapeutic challenge. In order to identify likely targets for therapy, it is important that the relative as well as the combined roles of these components are well understood. Studies employing the Zucker obese rat or the ob/ob mouse model are useful for outlining the overall outcomes of metabolic syndrome (73; 74), but do not assess the contribution of individual factors. Thus, we used the Type 2 diabetic GK rat, a lean and normotensive model, combined with diet-induced hyperlipidemia to study the effects of hyperglycemia and hyperlipidemia either alone or in combination. Although not overtly obese, the high-fat fed diabetic GK rat demonstrated elevated plasma levels of cholesterol, triglycerides and insulin. We did not observe a further elevation in blood pressure with high-fat diet treatment in both control and diabetic animals. Dobrian and colleagues reported in their rat model of diet-induced obesity, that the animals diverged into being obesity-prone (OP) or obesity-resistant (OR), and that only the OP animals developed hypertension (6). Thus, it will be interesting to understand potential mechanisms by which the GK rat remained resistant to development of hypertension.

Vascular remodeling in Type 2 diabetes has not been clearly understood, although there have been a few reports of hypertrophic microvascular remodeling in rodent models of Type 1 diabetes (14; 16). Using obese and insulin resistant rodent models, the role of the leptin receptor has been highlighted in modulating vessel

structure in diverse vascular beds (12; 73; 74). In our study, we observed that diabetes causes vascular remodeling by increasing M/L ratios, which was paralleled by an increase in adventitial collagen turnover. The addition of a high-fat diet did not further affect vascular structure (M/L) and collagen turnover. Expression of type 1 collagen was increased in hyperlipidemia in diabetes. When taken separately, both diabetes and diet-induced hyperlipidemia showed a strong trend for increased expression of collagen type 1, but statistical analysis showed only a significant interaction between diabetes and high-fat diet. Molnar et al. reported using a mouse model of diet-induced hyperlipidemia and Type 2 diabetes that although there was a worsening in cardiometabolic parameters, it did not cause vascular remodeling but lead to impaired endothelial-dependant vasodilation (13). Further studies in our animal model focusing on vasorelaxation and constriction pathways would clarify whether or not the structural and functional changes in microvessels are correlative.

Mechanical properties of the vessel are interdependent on vascular structure and the components of the extracellular matrix such as collagen, fibronectin and elastin. Intengan et al. reported in an animal model of hypertension that microvascular stiffness is altered, thus affecting the functional lumen diameter, total peripheral resistance, distensibility and compliance (19). They also established that the potent vasoconstrictor ET-1 is involved in mediating resistance artery stiffness in salt-sensitive experimental hypertension (49). In our animal model of Type 2 diabetes, there was a significant increase in plasma ET-1 levels, and high-fat diet feeding caused a further elevation, which was more pronounced in combined hyperglycemia and hyperlipidemia. The beta-coefficient, an indicator of vessel stiffness was higher in diabetes compared to control

animals. High-fat fed diabetic animals also showed increased vessel stiffness, but not higher than their diabetic counterparts on a normal diet. Thus, the elevated levels of ET-1 in diabetic rats on a high-fat diet may not necessarily correlate with vascular mechanics to the same degree, although there is a definite impairment. Vascular compliance (stiffness) also closely paralleled MMP-13 expression, which is involved breakdown of fibrillar collagen, promoting tissue remodeling (23). Myogenic tone was significantly higher in diabetic animals compared to controls. However, the hyperglycemia and high-fat diet combination decreased myogenic tone in the diabetic animals to match control values. Since myogenic tone is an indicator of vessel autoregulation, our data suggests a possibility of differential hemodynamics in diabetes and high-fat feeding. It is thus important to assess mesenteric blood flow in these subsets to ascertain the interaction between the two.

Lucchesi et al. reported that MMPs 2 and 9 were both involved in myogenic tone generation via a growth factor transactivation mechanism in mice mesenteric arteries (32; 80). Earlier studies from our lab established a role for the gelatinases in vascular remodeling in both the mesenteric and cerebral circulations (20; 21). In the current study, we observed alterations in the mechanical properties of the vessel but did not see any change in gelatinolytic activity. This difference could be attributed to variable factors such as the source of this particular subset of GK rats, blood glucose, body weights and blood pressure levels. Looking back at the metabolic profiles of the GK rats used in our earlier studies, the subjects used in the current study had comparably lower blood glucose levels (20; 21; 56). Although expression of MMPs 2 and 9 were increased with high-fat diet administration, TIMP-2 levels were increased only in combined

hyperglycemia and hyperlipidemia. Thus TIMP-2 maybe regulating MMP-2 activity in diabetic rats on a high-fat diet, but a different silencing mechanism could be involved in hyperlipidemia alone. Further studies need to be done to determine expression of MMPs 2 and 9, and to estimate the levels of TIMPs that endogenously inhibit MMP activity.

To summarize, in this study we sought to dissect the relative contributions of hyperglycemia and hyperlipidemia in mediating vascular remodeling and mechanical complications, and to examine the role of MMPs in extracellular matrix reorganization. While addition of a high-fat diet to diabetes did not alter vascular morphology, it had an impact on collagen turnover and collagenase expression, thereby affecting the mechanical properties of the vessel. There was a disease-diet interaction in causing increased vascular stiffness and altered myogenic reactivity in combined hyperglycemia and hyperlipidemia. It is thus important to target the individual components of metabolic syndrome to effectively prevent the development of cardiovascular disease.

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ACKNOWLEDGEMENTS

Table 3.1. Metabolic parameters in Type 2 diabetes and hyperlipidemia.

	Control	Control HF	Diabetic	Diabetic HF
Weight gain	87.7 ± 9.3	115.2 ± 13.4 *	69.8 ± 1.5	108.8 ± 8.5 **
Blood Glucose	99.1 ± 9.4	97.6 ± 5.9	158.7 ± 12.9 *	253.3 ± 32.1*,**
Blood Pressure	117.6 ± 3.1	121 ± 2.7	108.4 ± 1.5 *	111.6 ± 1.8*
Cholesterol	70.5 ± 4.6	72.1 ± 5.6	88.6 ± 3.5	123.4 ± 9.2*,**
Triglycerides	63.1 ± 2.6	87.0 ± 18.7	37.8 ± 0.5*	60.6 ± 8.1**
Insulin	1.5 ± 0.3	1.6 ± 0.5	1.2 ± 0.2	2.1 ± 0.2**
ET-1	0.43 ± 0.03	0.81 ± 0.23 *	1.43 ± 0.15 *	2.35 ± 0.23*,**
Leptin	117 ± 18	365 ± 50 *	268 ± 25 *	621 ± 118 *
HbA1C	5.4 ± 0.1	5.4 ± 0.2	7.6 ± 0.6*	10.1 ± 1.0 *,**
Adiposity	0.03 ± 0.002	0.05 ± 0.004*	0.03 ± 0.001	0.06±0.004*,**

*p<0.05 vs Control, **p<0.05 vs Diabetic (Mean ± SE; n=4-10/group)

(Units: Body weight, weight gain – g, Glucose – mg/dl, HbA1C - %, Blood Pressure (MAP) – mmHg, Insulin – μg/l, Cholesterol, Triglycerides – mg/dl, ET-1 – fmol/ml, Leptin - Adiposity (fat weight/body weight) – no unit).
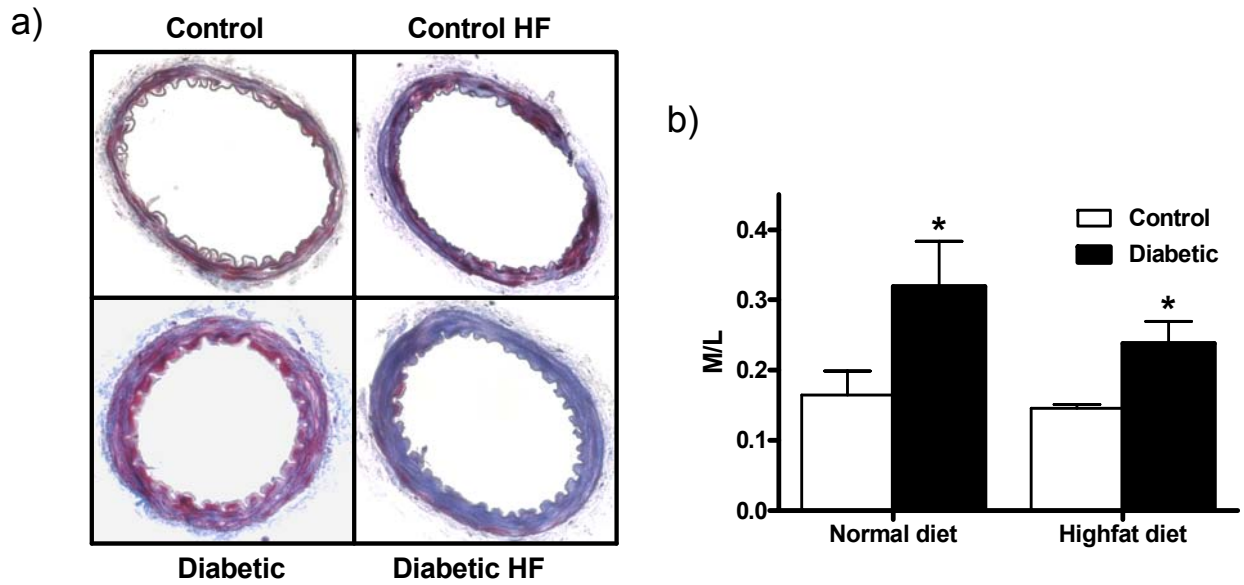
Figure 3.1

a)

b)

FIGURE 3.1. Vascular remodeling in Type 2 diabetes and hyperlipidemia. Formalin-fixed mesenteric artery cross-sections were analyzed for morphological changes by Masson staining.   Diabetes caused an increase in the media-to-lumen ratio (M/L), which was not affected by high-fat diet feeding in either control or diabetic animals. Representative images are shown in Panel A and combined analysis is given in Panel B. *p<0.05; n=5-8/group.

Figure 3.2
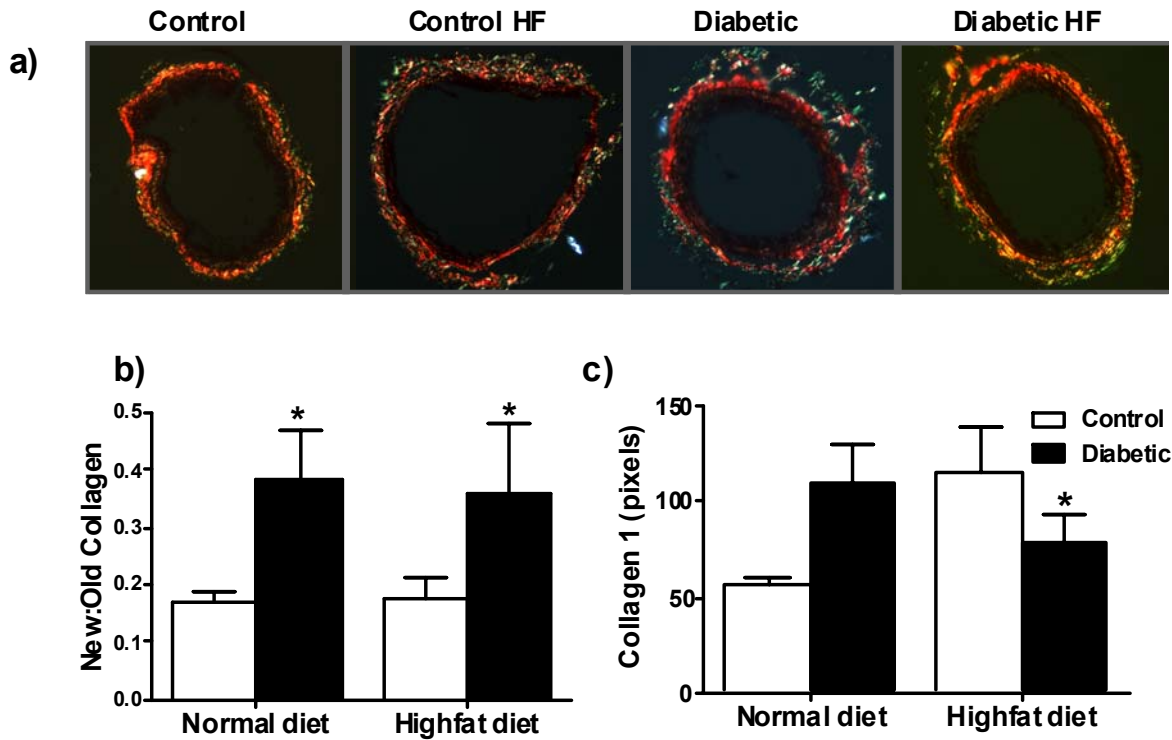
FIGURE 3.2. **Collagen deposition and turnover.** Collagen deposition patterns were observed from picrosirius red stained cross-sections, viewed under polarized light. There was increased collagen turnover in diabetes compared to control (increased green to red ratio in staining); addition of a high-fat diet did not have an effect on collagen regulation and turnover. Representative images are shown in Panel A and combined analysis is given in Panel B. Type 1 collagen expression is increased with high-fat diet treatment in diabetic animals. $*p<0.05$; n=3-5/group.

Figure 3.3
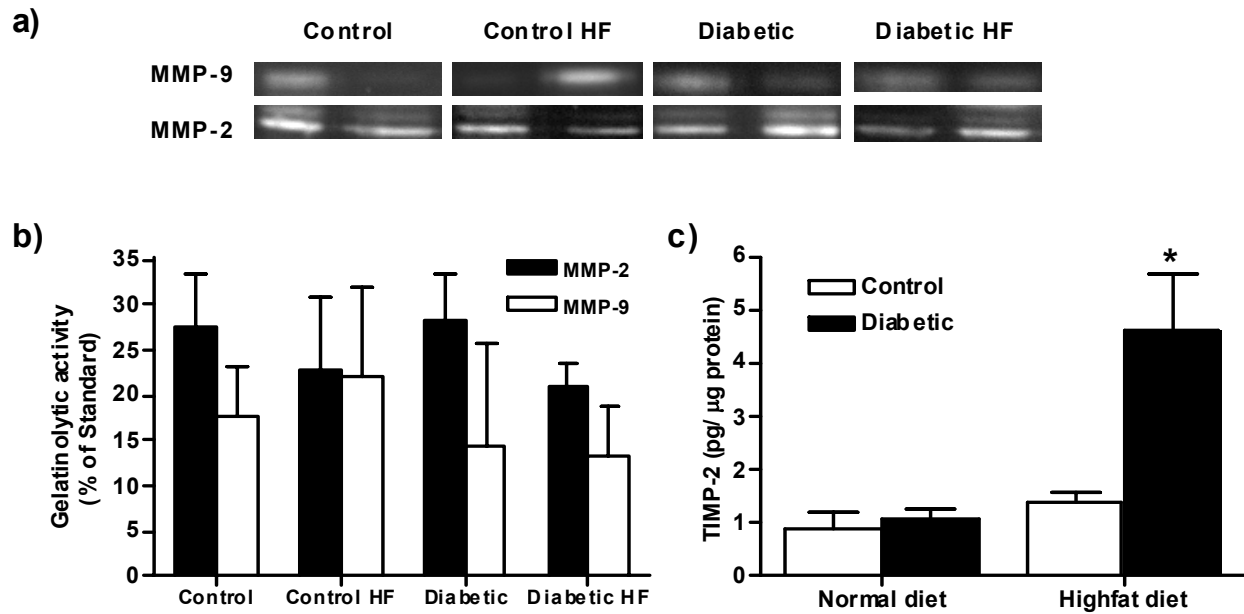
FIGURE 3.3. Gelatinolytic activity in Type 2 diabetes and hyperlipidemia.

Enzymatic activity of MMPs 2 and 9 was analyzed by gelatin zymography.

Representative images of lytic bands of MMPs 2 and 9 are shown in panel A.

Densitometric analysis of lytic bands for MMP-2 and 9 (panel B) revealed no differences

between groups. TIMP-2 levels were increased in diabetic rats that received a high-fat

diet (panel C). *p<0.05; n=4-6/group.

Figure 3.4
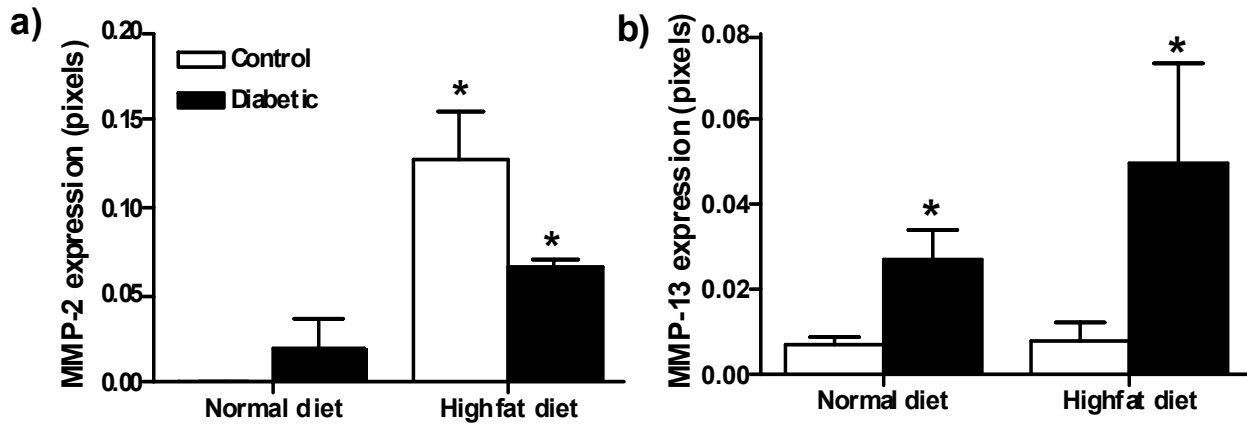
FIGURE 3.4. MMP expression in Type 2 diabetes and hyperlipidemia. **(A)** MMP-2 expression was increased with high-fat diet treatment. **(B)** Expression of MMP-13 was elevated in diabetic animals receiving either normal or high-fat diet. All densitometry was normalized using β-actin as a loading control. *$p < 0.05$; n=4-6/group.

Figure 3.5
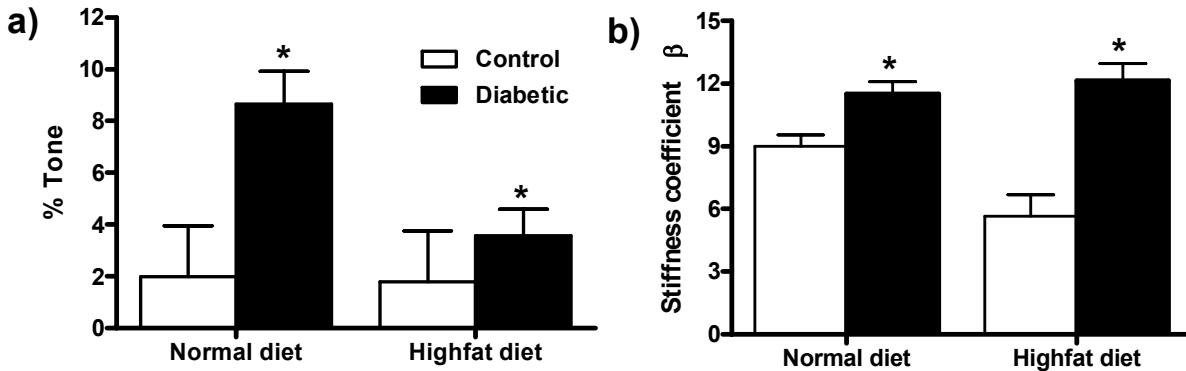
FIGURE 3.5. Mechanical properties in Type 2 diabetes and hyperlipidemia.

(A) Coefficient of stiffness or β-coefficient was increased in diabetes and in diabetic

animals on a high-fat diet. There was no effect of the high-fat diet on vessel stiffness in

controls. (B) Myogenic tone was assessed in the arteriograph at 60 mmHg.

Hyperglycemia increased microvascular tone in diabetes, thus impairing vessel

mechanics. High-fat diet treatment did not worsen mechanical properties in both control

and diabetic animals. *p<0.05; n=5-8/group.

CHAPTER 4

DIET-INDUCED HYPERLIPIDEMIA IMPAIRS MICROVASCULAR FUNCTION IN

TYPE 2 DIABETES [1]

ABSTRACT

Type 2 diabetes is often accompanied by co-morbid conditions such as obesity, insulin resistance and hypertension, clustering together to form the metabolic syndrome. In animal models of metabolic syndrome and insulin resistance, vascular dysfunction is known to exist, both due to increased constriction to vasoconstrictors and decreased relaxation by endothelial mechanisms. We have previously demonstrated that mesenteric arteries in Type 2 diabetes exhibit hyperreactivity to potent vasoconstrictor endothelin-1 (ET-1). Thus, we hypothesized that addition of a high-fat diet would exacerbate vascular dysfunction in resistance arteries in Type 2 diabetes. Third-order mesenteric arteries from control Wistar rats and Type 2 diabetic Goto-Kakizaki (GK) rats on either a normal or high-fat diet were mounted on a pressurized arteriograph and vascular function was studied. Maximum relaxation to acetylcholine (ACh, 0.1nM - 1µM) was decreased in combined hyperglycemia and hyperlipidemia, and so was the sensitivity to sodium nitroprusside (SNP, 0.1nM - 1µM). Hyperreactivity to ET-1 (0.1-100nM) was observed only in diabetic rats fed a high-fat diet. Expression of contractile proteins calponin and α-actinin in vascular smooth muscle cells was augmented in diabetic animals fed a high-fat diet, correlating with the vascular function studies. α-actinin levels were also elevated in control animals on a high-fat diet. Taken together, our results suggest that hyperlipidemia in Type 2 diabetes leads to microvascular dysfunction. There could be an association of VSMC contractile protein expression with vascular function. Further studies need to be done to elucidate the molecular mechanisms underlying the impaired vascular function.

INTRODUCTION

The vascular endothelium plays a prime role in regulating basal tone,
permeability, coagulation and VSMC growth (34). It is an early target for attack in
diabetes, and thus participates in the diabetic vascular disease process (35). The
production of vasoactive factors by the endothelium is in a delicate balance. However,
in diabetes, this ratio tends towards increased production of vasoconstrictors like
endothelin-1 (ET-1) and decreased vasodilators like nitric oxide (NO) and prostaglandin
I2 (36). Vascular dysfunction in microvessels is defined as hyperreactivity to
vasoconstrictors or impaired relaxation to vasodilators is associated with diabetes, and
in obesity, insulin resistance and hypertension that often present along with Type 2
diabetes (33). ET-1 is mitogenic in addition to being a potent vasoconstrictor, and has
been shown to mediate both vascular remodeling and dysfunction (37). Data from our
lab, as well as reports from others suggest a hyperreactive response of microvessels to
ET-1 in animal models of Type 2 diabetes or insulin resistance. Vascular dysfunction in
these models is also associated with impairment in specific endothelial pathways of
relaxation (38-40; 42). In the mesenteric arteries of the obese Zucker rat, a leptin-
deficient model of metabolic syndrome, impaired relaxation and constriction responses
have been reported (43), and this effect is also consistent with other vascular beds (81;
82). Molnar et al. reported impaired endothelium-dependent vasorelaxation in a mouse
model of diet-induced obesity (13). However, none of these studies demonstrate the
relative roles of hyperglycemia and hyperlipidemia in vascular pathophysiology.

Vascular function has been primarily linked to endothelial properties, whereas the
smooth muscle layer is more referred to in the context of vascular mechanics. In

skeletal muscle arteries of Type 2 diabetic mice, it has been shown that basal tone is increased thus affecting skeletal muscle blood flow (83; 84). Ito et al. reported increased myogenic reactivity in ophthalmic arteries in Type 2 diabetes (51; 52). Several proteins such as calponin, caldesmon and α-actinin have been shown to regulate the contractile properties of vascular smooth muscle cells (VSMC) by acting like molecular switches that favor the cell to stay in a contractile rather than a plastic phenotype (85). Both calponin and α-actinin are actin-binding proteins known to play a role in VSMC contraction by interacting with the actin-myosin apparatus in vascular smooth muscle cells, while caldesmon is involved in cell migration (86; 87). Calponin plays an important role in regulating the smooth muscle contraction in the mesenteric vessels, aorta and bladder (86; 88). However, the association of these contractile proteins vascular function in Type 2 diabetes is not well understood.

In this study, we examined the effect of diet-induced obesity on vascular function in Type 2 diabetes and on the expression of contractile proteins calponin and α-actinin. Our central hypothesis was that Type 2 diabetes would lead to vascular dysfunction of mesenteric microvessels, which would be worsened by the addition of a high-fat diet. Type 2 diabetic, non-obese and normotensive Goto Kakizaki (GK) rats (58) were used for the studies along with normoglycemic Wistar rats as controls.

METHODS

Animals and treatment

All experiments were performed on male control Wistar (Harlan, Indianapolis, IN) and diabetic GK (in-house bred, derived from the Tampa colony) rats. All protocols were

approved by the Institutional Animal Care and Use Committee (IACUC). The animals were housed at the Medical College of Georgia animal care facility, approved by the American Association for Accreditation of Laboratory Animal Care.

Four experimental groups were included in the study – control Wistar and diabetic GK rats on either a normal or a high-fat diet (the same quantity of a high-fat diet was administered to both strains of rats throughout the study period). The high-fat diet contained 36% fat (15.2% saturated and 20.8% unsaturated), 35% carbohydrates and 0.4% salt (Cat.# F2685, Bio Serv, NJ) (7; 8) and was given from week 11 through week 18. The control diet had 4.4% fat (2.5% saturated and 1.9% saturated) 46.6% carbohydrates and 0.3% salt.

Blood glucose was measured twice weekly from the tail-vain using a commercially available glucometer (Freestyle, Alameda, CA). Blood pressure (MAP, mmHg) was measured by tail-cuff plethysmography (Kent Scientific, Torrington, CT). MAP was recorded from week 8 through week 18, twice a week. This technique was validated on telemetry transmitter-implanted animals to yield comparable results (56). After week 18, animals were sacrificed and blood was collected in heparinized vials. Long term glucose control was assessed from glycosylated hemoglobin values (A1C, Metrika Inc., Sunnyvale, CA).

Surgical procedures

Animals were anesthetized with sodium pentobarbital and ex-sanguinated via cardiac puncture. The mesenteric bed was harvested, immersed in ice-cold Krebs-HEPES buffer and third-order mesenteric arteries were isolated for vascular function studies, while the remaining was snap-frozen for immunoblotting.

Vascular function studies

Mesenteric artery segments were mounted onto the pressurized arteriograph containing Krebs-HEPES buffer. The vessel chamber (CH1/QT) was placed under an inverted light microscope stage coupled to a video dimension analyzer (VDA, Living Systems Instrumentation, Burlington, VT). Oxygenated Krebs-HEPES buffer maintained at 37°C was continuously circulated through the vessel chamber. In addition, the lumen of the vessel was filled with the same buffer through the proximal cannula driven by a peristaltic pump and maintained at a constant pressure of 50. E*ndothelium-dependent and *independent relaxation* responses were determined using acetylcholine (ACh, 1nm-5μm) and sodium nitroprusside (SNP, 0.01-100μm) in vessels preconstricted with 5-HT to 70% over baseline. All reagents were added abluminally. Relaxation responses were calculated as a % change in lumen diameter from baseline. All responses were normalized to 5-HT preconstriction, which was taken as 100%. Dose-response curves were analyzed by curve-fitting (GraphPad software), and sensitivity EC_{50} (nM) and R_{max} (%) values were calculated to assess sensitivity and magnitude of responses.

Vasoconstriction to endothelin-1 (ET-1, 0.1-100nm) was similarly determined, except in this case, the vessels were equilibrated in a self-heating chamber (CH1-SH, Living Systems Instrumentation, Burlington, VT) regulated through a temperature controller, wherein, the buffer does not continually circulate through the chamber but is kept stationary. This method was employed in order to minimize the usage of ET-1. Vasoconstriction responses were normalized to baseline measurements taken before addition of ET-1.

Immunoblotting

Snap-frozen third-order mesenteric arteries were homogenized and immunoblotting was performed as previously described (20). Antibodies to determine the expression of calponin and α-actinin (C2687 and A7811, Sigma Aldrich, Saint Louis, MO) were used. Densitometric measurements were normalized using β-actin (A3854, Sigma Aldrich, Saint Louis, MO) as a loading control.

Statistical Analysis

A two-way ANOVA was performed comparing control and diabetic animals on normal or high-fat diet, in order to evaluate EC_{50} and R_{max} differences in vascular responses to ET-1, ACh and SNP. Nonlinear regression was used to plot relaxation responses to ACh and SNP. A sigmoidal dose-response curve was plotted to show vasoconstriction responses to ET-1. A one-way ANOVA for repeated measures was used to determine group differences across ET-1, ACh and SNP concentrations. Post-hoc group comparison at each concentration was done using a Bonferroni adjustment for the multiple comparisons. Results are reported as unadjusted mean ± SE. Statistical significance was determined at $p < 0.05$. Graphpad Prism 5.0 was used for all analyses and graphing (Graphpad software, San Diego, CA).

RESULTS

Metabolic parameters

Weight gain was calculated as a difference in body weight of the animals at the end and beginning of the high-fat diet administration period (corresponding time points were used in groups receiving normal diet). This was higher in control animals on

normal diet when compared to their diabetic counterparts. The high-fat diet caused an increased in body weight in both control and diabetic animals to a similar degree. Blood glucose was higher in diabetic animals, and administration of the high-fat diet elevated it nearly two-fold. However, control animals receiving high-fat diet did not have increased blood glucose levels. These measures were paralleled by glycosylated hemoglobin measures (Hb A1C) that were significantly elevated with and without a high-fat diet in Type 2 diabetes. Blood pressure (MAP) was slightly elevated in controls compared to diabetic animals. (Table 4.1).

Vascular function

Vasoconstrictor responses to ET-1 were examined in all four groups of animals. Combined hyperglycemia and hyperlipidemia (diabetic HF group) caused hyperreactivity to ET-1, as was seen with the increased R_{max} of this group compared to the other groups. There was no change in the sensitivity (EC_{50}) to ET-1 between groups. (Figure 4.1, Table 4.2).

Endothelium-dependent relaxation to ACh was comparable between control and diabetic animals of normal diet. Addition of a high-fat diet impaired ACh-mediated vasorelaxation in diabetes alone, with a decrease in R_{max} and inability of the vessels to relax back to baseline. The high-fat diet did not affect maximum relaxation to ACh in control animals. There was no difference in sensitivity to ACh across groups. (Figure 4.2a, Table 4.2).

SNP was used to test endothelium-independent mechanisms of vasorelaxation. There was a decreased sensitivity to SNP in diabetic animals on a high-fat diet

compared to the other groups. Maximum relaxation was not affected. (Figure 4.2b, Table 4.2).

Expression of contractile proteins

Immunoblotting was performed to determine the expression of α-actinin and calponin. α-actinin was upregulated upon addition of a high-fat diet in both control and diabetic groups, but there was no additional effect of diabetes on its expression (Figures 4.3a and b). Calponin levels were elevated with high-fat diet treatment in diabetes alone, while the other groups had comparable levels (Figures 4.3a and c).

DISCUSSION

This study examined the mechanisms of vascular function in mesenteric microvessels in Type 2 diabetes, with or without the additional effects of diet-induced hyperlipidemia, and its impact on the expression of VSMC contractile proteins calponin and α-actinin. The major findings of this study are: 1) there was no hyperreactivity to the potent vasoconstrictor ET-1 or impaired relaxation to vasodilators in Type 2 diabetic animals compared to controls, 2) combined hyperglycemia and hyperlipidemia caused impaired relaxation responses to both endothelium-dependent and independent vasodilators, and there was an exaggerated constriction response to ET-1, 3) there is increased expression of the actin-binding protein calponin with high-fat diet administration in Type 2 diabetes while diet-induced hyperlipidemia is associated with increased expression of α-actinin.

Vascular dysfunction has been elucidated in animal models of metabolic syndrome, insulin resistance and in chemically induced models of Type 1 diabetes to a

considerable degree, but there is still a paucity of studies in Type 2 diabetes (33; 40-43; 81; 82; 89). The zucker obese rats and the db/db mice serve as good models to study the effects of diabetes, hypertension and obesity in unison, but their relative contributions cannot be dissected. The Goto Kakizaki rat, a spontaneous model of Type 2 diabetes and being non-obese and normotensive is thus a good model to study the individual as well as combined roles of hyperglycemia and hyperlipidemia, which can be induced with a high-fat diet.

Prior to our study, the role of ET-1 in mediating vasoconstriction has been demonstrated by several groups including our own (37; 39; 42). Previously, we reported that there is an upregulation of the ET system in Type 2 diabetes leading to hyperreactivity of mesenteric microvessels (39). However, we could not reproduce those observations in our current study. One possible explanation for this variability is the difference in techniques involved to assess microvascular function. In our earlier study we employed a non-perfused artery preparation with the wire-myograph, in which there are no interfering effects of intraluminal flow and transmural pressure. The vessel is tensioned to a point where is it expected to produce maximum responses to vasoactive agents. However, this is not the case in the arteriograph where the vessel is maintained at a constant perfusion pressure and there is no effect of tension. Further, in the analysis of data using the myograph, vascular responses are expressed as the amount of force generated, thus taking into account the dynamics of the vessel wall. However, data from the arteriograph are expressed as a percent change in lumen diameter from the baseline. Edvinsson and colleagues recently reported vascular responses in the rat middle cerebral artery using perfused and non-perfused

approaches to a similar effect. With the wire-myograph, they reported more than a two-fold increase in maximum responses to vasoactive agents in cerebral vessels when compared to those mounted on the perfused arteriograph (90; 91). Another factor to be considered is the blood glucose profiles of the animals in the study, as the intensity of hyperglycemia could have a direct correlation to the degree of vascular dysfunction. The subset of diabetic animals used in our earlier study had higher levels of blood glucose than those considered in the current study (39).

ACh-induced endothelial-dependant vasorelaxation was decreased in combined diabetes and hyperlipidemia, consistent with results from a similar study by another group that employed a mouse model of diet-induced obesity and Type 2 diabetes (13). We also found impaired non-endothelial relaxation in mesenteric microvessels of Type 2 diabetic rats fed a high-fat diet. The VSMC layer plays an important role in regulating the mechanical properties of the vessel. In our animal model, we previously demonstrated that Type 2 diabetes leads to vascular remodeling and impaired function in both the mesenteric and cerebral circulations (20; 21; 39). Vascular structure and mechanical properties are closely correlated. Myogenic tone is the intrinsic ability of the vessel to respond to hemodynamic stress. Ito and colleagues reported decreased myogenic reactivity of ophthalmic arteries of Type 2 diabetic rats in response to acute high glucose concentrations (51; 52). However the association between vascular dysfunction and mechanical properties in resistance arteries needs to be established in our Type 2 diabetic model. Furthermore, changes in total peripheral resistance and function need to be correlated to differences in blood flow in the mesenteric circulation.

Cytoskeletal proteins α-actinin and calponin mediate VSMC contraction by binding to the actin-myosin apparatus. Both these contractile proteins are known to be upregulated with hyperglycemia and hyperinsulinemia *in vitro* (92; 93). Prados et al. reported high circulating levels of α-actinin in diabetic patients (94), but the association of microvascular dysfunction in Type 2 diabetes to the expression of these contractile proteins was not well understood. We observed increased expression of calponin and α-actinin in mesenteric microvessels in combined hyperglycemia and hyperlipidemia, where vascular dysfunction occurs via both endothelial and non-endothelial mechanisms. Further studies on these contractile proteins need to be done in order to define their role in mediating vascular function in Type 2 diabetes.

In summary, we hypothesized that Type 2 diabetes would lead to vascular dysfunction in mesenteric microvessels and the addition of a high-fat diet would cause further worsening. From this study, we conclude that combined hyperglycemia and hyperlipidemia leads to heightened vasoconstriction and decreased relaxation, associated with increased expression of contractile proteins calponin and α-actinin.

ACKNOWLEDGEMENTS

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Table 4.1: Metabolic parameters in Type 2 diabetes and hyperlipidemia.

	Control	Control HF	Diabetic	Diabetic HF	
Weight Gain (g)	87.7 ± 9.3	120 ± 8.8	66.9 ± 2.5	95.9 ± 5.5	C>D, HF>N
Blood Glucose (mg/dl)	121 ± 10	99 ± 3	157 ± 12	291 ± 37	D>C, HF>N*
Hb A1C (%)	5.4 ± 0.1	5.4 ± 0.2	7.6 ± 0.6	10.1 ± 1	D>C, HF>N*
MAP (mmHg)	128 ± 3	123 ± 3	116 ± 3	112 ± 1	C>D

C – Control, D – Diabetes, N – Normal diet, HF – High fat diet

n=6-11/group; '*' represents an interaction between disease and diet obtained by two-way ANOVA. Data are presented as Mean ± SE.

Table 4.2: Vascular function in Type 2 diabetes and hyperlipidemia.

		Control	Control HF	Diabetic	Diabetic HF
ET-1	EC_{50}	3.4 ± 1.5	1.9 ± 0.1	2.1 ± 0.7	3.5 ± 0.7
	R_{max}	55 ± 6	59 ± 5	58 ± 5	74 ± 5 *
ACh	EC_{50}	8.1 ± 1.8	1.7 ± 1.5	6.0 ± 1.9	6.1 ± 1.9
	R_{max}	97 ± 3	96 ± 3	97 ± 2	74 ± 6 *
SNP	EC_{50}	5.7 ± 2	3.5 ± 1.2	6.5 ± 2.3	23.5± 10.4*
	R_{max}	102 ± 3	101 ± 2	99 ± 1	98 ± 2

Sensitivity (EC_{50}, nM) and magnitude (R_{max}, %) of vascular responses to ET-1, ACh and

SNP in mesenteric arteries of control Wistar and diabetic GK rats with or

without high fat (HF) diet administration.

'*' represents an interaction between disease (diabetes) and diet (high-fat diet) obtained

by two-way ANOVA. ($p < 0.05$); n = 5-6 per group. Data are presented as Mean ± SEM.

Figure 4.1
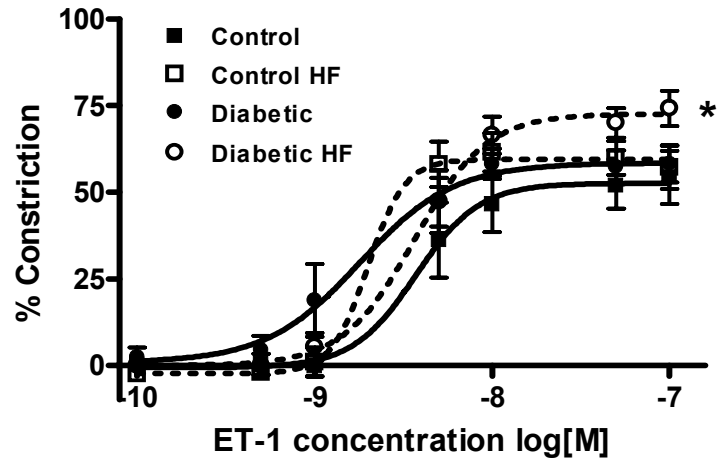
Figure 4.1: Vascular responses to ET-1. Vasoconstriction to ET-1 (0.1-100 nm) in resistance arteries of control Wistar and diabetic GK rats on normal or high-fat diet. Maximum constriction was increased in only diabetic rats fed the high-fat diet, and sensitivity to ET-1 remained the same across groups. *$p<0.05$ vs. control; n=5-6 per group. Open squares or circles with broken lines represent the high-fat diet treated groups, whereas filled squares or circles with full lines represent groups on normal diet.

Figure 4.2

Figure 4.2: Vascular responses to ACh and SNP. Endothelium-dependent and independent vasorelaxation responses to **(a)** ACh and **(b)** SNP in control Wistar and diabetic GK rats on normal or high-fat diet. Maximum relaxation to ACh was impaired in diabetic rats fed the high-fat diet, as was the sensitivity to SNP seen by the rightward shift in the curve representing 'Diabetic HF'. *$p < 0.05$ vs. control; n=5-8 per group. Open squares or circles with broken lines represent the high-fat diet treated groups, whereas filled squares or circles with full lines represent groups on normal diet.
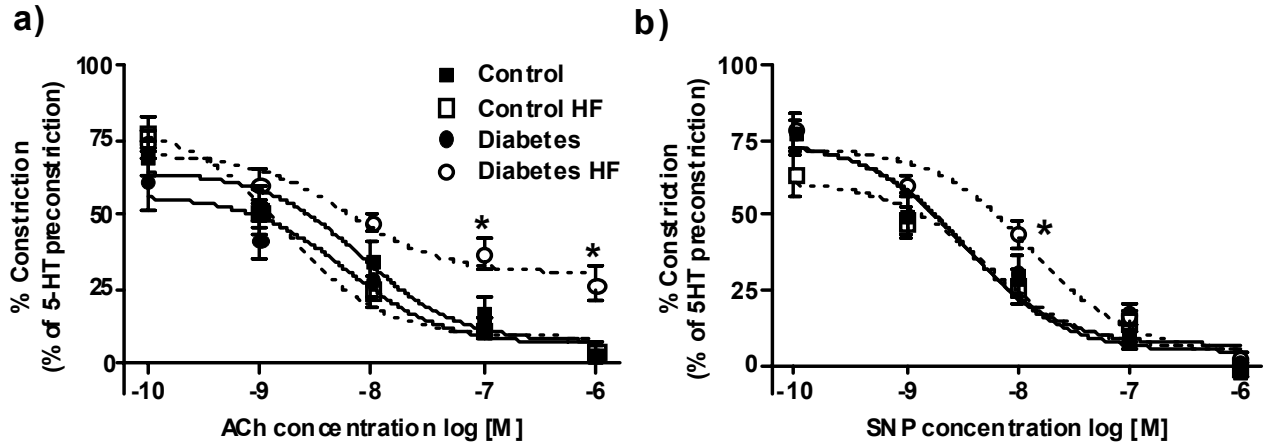
Figure 4.3
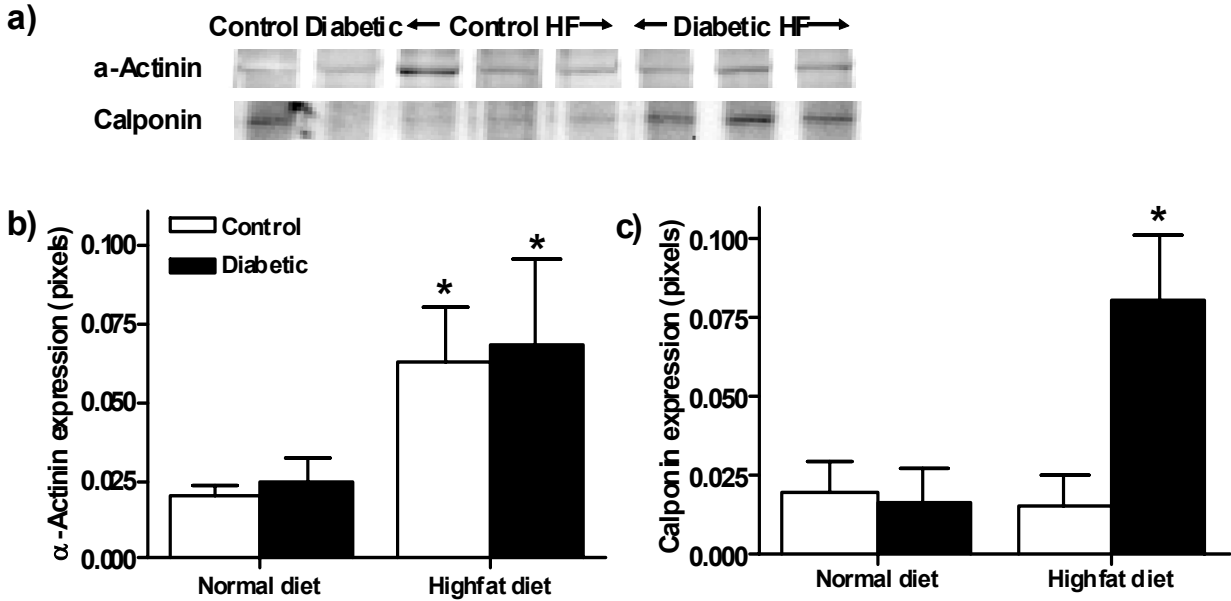
Figure 4.3: Expression of calponin and α-actinin. (a) Expression of contractile proteins and densitometric analyses of **(b)** α-actinin and **(c)** calponin. Protein levels of α-actinin were elevated with high-fat diet administration in both control and diabetic animals. Calponin expression was increased in diabetic animals fed high-fat diet. Densitometry values reported have been normalized to β-actin levels in all samples to account for differences in loading. *$p < 0.05$ vs. control; n=5-6 per group.

CHAPTER 5

DISCUSSION

The aims of the current study were to determine the effect of hyperglycemia due to Type 2 diabetes and the additional effect of diet-induced hyperlipidemia on vascular remodeling, altered mechanical properties and vascular dysfunction in mesenteric microvessels of Goto Kakizaki rats. Previous studies from our lab have demonstrated that there is significant medial thickening and collagen deposition mediated by increased MMP activity and an increase in ET-1 mediated vasoconstriction in third-order mesenteric arteries (21; 39).

Results from our current study suggest that prevention of vascular remodeling (Figures 2.1, 2.3 and 2.4), increased stiffness and myogenic tone (Figure 2.4) are probable mechanisms by which glycemic control using metformin offered vascular protection in mesenteric microvessels in Type 2 diabetes. Contrary to our hypothesis, diet-induced hyperlipidemia did not further augment vascular remodeling (Figures 3.1, 3.2 and 3.3) and impaired mechanical properties (Figure 3.5) observed in Type 2 diabetes. However, combined hyperglycemia and hyperlipidemia impaired relaxation responses to different vasodilators (Figure 4.2) and increased vasoconstriction to ET-1 (Figure 4.1), which was not observed with hyperglycemia alone. These studies provide evidence that vascular complications can be manifested by Type 2 diabetes either alone or in combination with diet-induced hyperlipidemia, although varying in pathophysiology.

It was hypothesized that glycemic control would improve vascular function and compliance and decrease remodeling in mesenteric microvessels mediated by Type 2 diabetes, whereas it would be exacerbated by diet-induced hyperlipidemia. We proposed to study the regulation of MMPs, zinc-dependant proteolytic enzymes responsible involved in extracellular (ECM) matrix turnover as a mechanistic target in diabetes-mediated pathophysiology. One major finding of the study was that glycemic control by administration of metformin was effective in preventing vascular remodeling observed during diabetic progression. At 18 weeks of age (Diabetic 18wk group), there was a significant increase in M/L due to mild-to-moderate hyperglycemia. The diabetic subset that was administered metformin shortly after elevation of blood glucose (>150 mg/dl) showed no vascular remodeling (Figure 2.1, Table 2.2). Collagen turnover that was assessed by picrosirius red staining also followed a similar pattern, wherein, metformin treatment prevented the increased turnover seen in the diabetic rats, suggesting the beneficial effects of glycemic control initiation early on during diabetic progression (Figure 2.3). With diabetic progression, an outward hypertrophic remodeling was observed, with an increase in total vessel as well as lumen diameters, and a corresponding increase in cross sectional area of the vessels, without a change in M/L or a further increase in collagen turnover (Figure 2.2). Expression of collagen type 1, the most abundant vascular collagen was increased in the diabetic subsets at 10 and 18 weeks, but not at 24 weeks suggesting temporal differences in collagen synthesis and degradation, whereas metformin administration was associated with decreased collagen-1 levels (Figure 2.6).

In our earlier studies, a direct link between MMP regulation and vascular remodeling was established in both the middle cerebral and mesenteric arteries of Type 2 diabetic GK rats (20; 21). It was reported that an increase in gelatinolytic activity was a key mechanism by which collagen deposition and turnover were regulated in the vasculature. However in the current study, there were no differences in MMP regulation between diabetic rats at different ages and controls, as well as the diabetic rats that were made euglycemic (Figure 2.5). Interestingly, the blood glucose profiles of the diabetic animals used in the current study were lower than in those used in our earlier studies (Table 2.1), suggesting that although mild hyperglycemia was sufficient to promote vascular remodeling in mesenteric microvessels, it was probably not enough to show divergence in MMP regulation between groups.

A second major finding was that mechanical properties of the vessel were impaired in the diabetic 18wk subset, but not in the other diabetic groups reflecting on the dynamics of the vascular remodeling process. Promoting euglycemia using metformin also protected from impairment in vascular mechanics, highlighting again that the adverse effects on vascular structure and mechanics were mediated by hyperglycemia in Type 2 diabetes (Figure 2.4, Table 2.2). Su et al. defined a role for gelatinases, especially MMP-9 in increasing vasomotor tone, which was not observed when the MMP-9 gene was absent (32). However, in our study, changes in myogenic tone and stiffness in diabetes did not suggest a role for MMPs. Expression of MMP-13 paralleled increases in stiffness and myogenic tone (Figure 2.6). Further studies highlighting collagenase activity and their regulation by intrinsic modulators such as TIMPs and other MMPs need to be done in order to establish a role for MMP-13 in

mediating vascular mechanics. Neither vasoconstriction to ET-1 nor vasodilation to ACh and SNP were impaired in the GK rat, thus making it difficult to demonstrate the beneficial effect of glycemic control in Type 2 diabetes. Katakam et al. reported using a fructose-fed rat model of insulin resistance that administration of metformin improved endothelium-dependant vascular function (69). Earlier studies from our own lab showed that the ET system was upregulated in the GK rat, with an exaggerated response to ET-1 (39). Although our current approach in evaluating vascular function using the arteriograph did not establish a role for glycemic control in vascular function, one cannot rule out its likely beneficial effects.

As an extension to the first arm, we further hypothesized that addition of a high-fat diet would exacerbate impairments in vascular structure, mechanical properties and vascular function mediated by Type 2 diabetes. Diet-induced hyperlipidemia is commonly associated with neointimal formation in mice (12; 13). However, contrary to our hypothesis and published reports, the high-fat diet neither caused a further increase in M/L in diabetes, nor did it trigger proliferation in the neointima (Figures 3.1, Table 3.2). Expression of collagen type 1 was elevated with diet-induced hyperlipidemia in diabetic animals (Figure 3.2). Collagen turnover patterns that were visualized by picrosirius red staining also followed a similar trend (Figure 3.2). As discussed earlier, we demonstrated in our previous studies that MMP enzymes played a key role in regulating changes in vascular structure (20; 21). Although we observed vascular remodeling in diabetes alone, without an additional effect of a high-fat diet, these effects were not associated with activity of gelatinase enzymes MMPs 2 and 9 (Figures 3.3),

although control and diabetic animals on the high-fat diet showed differential regulation of MMP-2 by its endogenous inhibitor TIMP-2 (Figures 3.3 and 3.4).

Vascular mechanical properties also followed a similar pattern. Although Type 2 diabetes induced an increase in myogenic tone and vessel stiffness, diet-induced hyperlipidemia did not cause further impairment (Figures 3.5, Table 3.2). These results were paralled with an increase in MMP-13 expression in diabetes, either alone or in combination with a high-fat diet (Figure 3.4). Thus, another major conclusion of the current study was that diet-induced hyperlipidemia did not worsen vascular remodeling and mechanical properties in the GK rat model of Type 2 diabetes, although it caused an adverse elevation in metabolic parameters (Table 3.1). However, further studies are warranted using other experimental diets with varying abilities of inducing atherosclerotic changes and vascular remodeling, and longer durations of diet-administration before ruling out the adverse effects of diet-induced hyperlipidemia in Type 2 diabetes.

The fourth important finding of this study was the vascular function, which was not affected by Type 2 diabetes showed impairment in combined hyperglycemia and hyperlipidemia. Diet-induced hyperlipidemia caused increased constriction of resistance vessels to ET-1 in diabetes (Figure 4.1, Table 4.2), and it also decreased vasorelaxation mediated by both endothelial and non-endothelial mechanisms (Figures 4.2, Table 4.2). Vascular dysfunction was associated with an increase in expression of contractile proteins calponin and α-actinin (Figure 4.3). However, vascular dysfunction in combined Type 2 diabetes and diet-induced hyperlipidemia was not associated with impairments in vascular structure or mechanical properties. Similar to our observations, Molnar et al.

reported vascular dysfunction in their mouse-model of diet-induced obesity and Type 2 diabetes, in the absence of vascular remodeling and neointima formation (13). Thus, although both vascular remodeling and dysfunction have been observed in our Type 2 diabetic GK rat as well as in other animal models, the two anomalies do not always coexist (21; 37; 39; 72; 73).

FUTURE DIRECTIONS

Blood flow is an important physiological component that has not been addressed in the current studies. Several groups have demonstrated the changes in blood flow alone can initiate vascular remodeling in microvessels (37; 73-75). Thus, further studies are necessary to elucidate possible differences in mesenteric blood flow in order to correlate the vascular structure, function and mechanical properties studied thus far.

We anticipated that diet-induced hyperlipidemia would elevate blood pressure in Type 2 diabetes. However, the diabetic subsets in this study had lower blood pressure levels compared to their controls. Dobrain et al. reported that obesity induced by high-fat feeding leads to hypertension; however the subjects diverged into obesity-prone (OP) and obesity-resistant (OR) groups and only the OP animals would become hypertensive (6; 76; 77). It is possible that the GK rats are similar to the 'OR' animals in being resistant to blood pressure elevation, and it would be interesting to delve into potential mechanisms by which they regulate blood pressure.

The mesenteric circulation is not a special circulation in terms of autoregulation, although it does possess a certain degree of myogenic tone. It will be interesting to correlate these observations with parallel studies in cerebral, cardiac or renal

circulations to better understand the effects of Type 2 diabetes either alone, or in combination, on vascular outcomes. Further, a more detailed study on the regulation on contractile proteins such as calponin and α-actinin would help us better understand their role in mediating vascular function, and whether their upregulation in combined hyperglycemia and hyperlipidemia is a cause for vascular dysfunction or a mere consequence. Also, studies on vascular function with other vasoconstrictors such as norepinephrine and serotonin, and blockade of specific pathways mediating vasorelaxation such as nitric oxide, cyclooxygenase and endothelium-derived hyperpolarizing factors will provide us with selective targets for improving vascular function.

CLINICAL IMPLICATIONS

Impairment in endothelial function is an early event in the atherosclerotic process, and markers of endothelial dysfunction have oftentimes correlated with disease activity. For example, abnormal endothelial function in coronary arteries may precede the development of angiographically evident coronary plaques, and thus maybe used to predict future cardiovascular events. However, direct assessment of vasomotor responses in the coronary, cerebral or renal circulation may be invasive and cannot be widely applied in clinical practice. Endothelial dysfunction is considered a systemic process; thus endothelial-dependant function detected in peripheral arteries with non-invasive tests reflects endothelial function in these special circulations (95). Therefore, the studies presented in this dissertation need to be envisioned and

extrapolated in a much larger sense and in the context of clinical Type 2 diabetes and

hyperlipidemia, and their net effect on cardiovascular disease.

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