Differential Effects of Diet-Induced Dyslipidemia and Hyperglycemia on Mesenteric Resistance Artery Structure and Function in Type 2 Diabetes

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ABSTRACT

Type 2 diabetes and dyslipidemia oftentimes present in combination. However, the relative roles of diabetes and diet-induced dyslipidemia in mediating changes in vascular structure, mechanics, and function are poorly understood. Our hypothesis was that addition of a high-fat diet would exacerbate small artery remodeling, compliance, and vascular dysfunction in type 2 diabetes. Vascular remodeling indices [media/lumen (M/L) ratio, collagen abundance and turnover, and matrix metalloproteinase dynamics], mechanical properties (vessel stiffness), and reactivity to pressure and vasoactive factors were measured in third-order mesenteric arteries in control Wistar and type 2 diabetic Goto-Kakizaki (GK) rats fed either a regular or high-fat diet. M/L ratios, total collagen, and myogenic tone were increased in diabetes. Addition of the high-fat diet altered collagen patterns (mature versus new collagen) in favor of

matrix accumulation. Addition of a high-fat diet caused increased constriction to endothelin-1 (0.1–100 nM), showed impaired vasorelaxation to both acetylcholine (0.1 nM–1 μ M) and sodium nitroprusside (0.1 nM–1 μ M), and increased cardiovascular risk factors in diabetes. These results suggest that moderate elevations in blood glucose, as seen in our lean GK model of type 2 diabetes, promote resistance artery remodeling resulting in increased medial thickness, whereas addition of a high-fat diet contributes to diabetic vascular disease predominantly by impairing vascular reactivity in the time frame used for this study. Although differential in their vascular effects, both hyperglycemia and diet-induced dyslipidemia need to be targeted for effective prevention and treatment of diabetic vascular disease.

It is estimated that over 23 million Americans are affected by type 2 diabetes. Obesity, insulin resistance, and hypertension often cluster along with type 2 diabetes, resulting in a condition known as metabolic syndrome or syndrome X, thus imposing an enormous task from a therapeutic standpoint (Muhammad, 2004). Although studies with obese Zucker rats (Frisbee, 2003; Stepp et al., 2004; Bouvet et al., 2007) and ob/ob mice (Schäfer et al., 2004) provide important evidence about complications of obesity, insulin resistance, hypertension, and prediabetes in the systemic microvasculature, the relative contributions of the individual components of metabolic syndrome to diabetes-associated complications cannot be dissected. Furthermore, effects of hyperglycemia and

high-fat diet on the structure and mechanics of contractile medial layer are less understood. To define specific targets and develop therapeutic strategies to treat vascular complications, it is very important that the relative contributions of hyperglycemia and hyperlipidemia to vascular structure and function independent of atherosclerotic changes are well defined. The Goto-Kakizaki (GK) rat, being a spontaneous model of type 2 diabetes without the presence of comorbid complications, thus offers an excellent opportunity to study the individual role of mild to moderate hyperglycemia (which is the case in a vast majority of type 2 diabetic patients) in mediating vascular complications and also its effects in combination with high-fat diet.

Changes in the structure of small arteries in the streptozotocin-induced type 1 diabetes model are characterized by medial thickening and decreased diameter of the lumen, thus increasing the media/lumen (M/L) ratio, an index of vascular remodeling (Cooper et al., 1994, 1997). We have shown re-

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ABBREVIATIONS: GK, Goto-Kakizaki; M/L, media/lumen; ET, endothelin; MMP, matrix metalloproteinase; ECM, extracellular matrix; Ach, acetylcholine; SNP, sodium nitroprusside; TIMP, tissue inhibitor of MMP; ANOVA, analysis of variance; HF, high fat.

cently that vascular remodeling also occurs in GK rats, and endothelin (ET)-1, a potent vasoconstrictor with profibrotic properties, mediates this effect. In this model, there is an up-regulation of the matrix metalloproteinase (MMP) system in both the systemic and cerebral circulations, contributing to vascular remodeling (Harris et al., 2005; Sachidanandam et al., 2007). The MMPs are a family of zinc-dependent enzymes that are extensively involved in vascular remodeling by regulating extracellular matrix (ECM) turnover in a very complex manner (Nagase and Woessner, 1999). However, the role of MMPs in mediating vascular remodeling in combined hyperglycemia and hyperlipidemia is unknown.

Mechanical properties of the vessel dictate its compliance and adaptability to changes in pressure and shear stress. Vessel stiffness governs distensibility and compliance in response to a dynamic environment, whereas myogenic tone denotes the intrinsic ability of the vascular smooth muscle cells to respond to changes in pressure and hemodynamic stress (Coulson et al., 2002). Data from our laboratory suggests that there is significant medial thickening and collagen deposition in mesenteric resistance arteries in type 2 diabetes (Sachidanandam et al., 2007), but the relationship of these changes to vascular mechanical properties has not been studied. In addition to myogenic tone, reactivity of blood vessels to vasoactive agents is important in regulation of vascular function. Past studies 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 (Katakam et al., 2001; Miller et al., 2002; Brondum et al., 2005; Sachidanandam et al., 2006). Given that actin-binding proteins such as calponin and α -actinin regulate the contractile properties of vascular smooth muscle cells by acting like molecular switches that favor the cell to stay in a contractile rather than a plastic phenotype (Lindqvist et al., 2001), the relative effect of hyperglycemia and a diet rich in fat content on the expression of these contractile proteins is important for regulation of vascular function.

Based on these past observations, we sought to examine the relative roles of hyperglycemia and diet-induced dyslipidemia in resistance small artery remodeling, compliance, and function and the involvement of the extracellular matrix in mediating these processes. Our central hypothesis was that a high-fat diet would exacerbate structural remodeling, impaired mechanical properties, and vascular dysfunction in mesenteric arteries of nonobese and normotensive, type 2 diabetic GK rats.

Materials and Methods

Animals and Diet. All experiments were performed on male control Wistar (Harlan, Indianapolis, IN) and diabetic GK (in-house bred, derived from the Tampa colony) rats (Standaert et al., 2004). The animals were housed at the Medical College of Georgia animal care facility, and all protocols were approved by the Institutional Animal Care and Use Committee. Four experimental groups were included in the study, a control Wistar and diabetic GK group and a control and diabetic group that were 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 (Bio-Serv, Frenchtown, NJ) (Wang et al., 2003; Zhou et al., 2005), and a control diet that had 4.4% fat (2.5%

saturated and 1.9% saturated), 46.6% carbohydrate, and 0.3% salt. The onset of diabetes in GK rats was around weeks 6 to 7, and a high-fat diet was given from weeks 11 through 18. All animals were placed in metabolic cages for a 24-h period at the beginning of treatment, and food and water intake was monitored. Blood glucose was measured twice weekly from the tail vein using a commercial glucometer (Freestyle; Abbott Diabetes Care, Inc., Alameda, CA). Long-term glucose control was assessed from glycosylated Hb values (A1C; Thermo Fisher Scientific, Waltham, MA). Blood pressure was recorded twice weekly using the tail-cuff method (Kent Scientific, Torrington, CT). At 18 weeks of age, animals were anesthetized with sodium pentobarbital and exsanguinated via cardiac puncture; blood was collected in heparinized vials and processed for plasma analyses. The mesenteric bed was harvested, immersed in ice-cold Krebs-HEPES buffer (calcium-free), and third-order mesenteric arteries were isolated. Adipose tissue from the perirenal and epididymal depots was collected and weighed. Plasma ET-1, insulin, triglycerides, and cholesterol were measured as described previously (Harris et al., 2005).

Vascular Structure. Third-order mesenteric arteries were fixed in formalin using the quick-transfer freezing chamber (Living Systems Instrumentation, Burlington, VT), wherein they were perfused (at 50 mm Hg for 30 min) and fixed in formalin simultaneously. This prevents variability that occurs with manual perfusion. Images were captured and wall thickness, lumen, and outer diameter were measured from Masson stained cross-sections using SPOT software (Diagnostic Instruments, Inc., Sterling Heights, MI).

Collagen deposition patterns were evaluated in mesenteric artery cross-sections stained with picrosirius red captured under polarized light as described previously (Said and Motamed, 2005). Mature collagen stained red or orange, whereas newly formed collagen stained green or yellow. Total collagen was quantified using Metamorph software (Molecular Devices, Sunnyvale, CA) by measuring the intensities of green- and red-stained regions. Collagen type 1 (Calbiochem, San Diego, CA) expression was evaluated by slot-blot analysis as described previously (Sachidanandam et al., 2007). Protein levels were normalized using β -actin (Sigma-Aldrich, St. Louis, MO) as a loading control.

Vascular Mechanics and Function. Mechanical properties of third-order mesenteric artery segments were studied in a small vessel arteriograph (Living Systems Instrumentation) as described previously (Rigsby et al., 2007). In brief, after a 30-min equilibration period at 50 mm Hg pressure, pressure-dependent (5–120 mm Hg) changes in lumen diameter were measured under active (Ca^{2+} -containing Krebs-HEPES buffer) and passive (Ca^{2+} -free Krebs-HEPES buffer) conditions. Myogenic tone, stress, strain, and stiffness (β -coefficient) were calculated using earlier reports. Stress-strain curves were plotted using KaleidaGraph version 4.0 (Abelbeck/Synergy, Reading, PA). The stiffness coefficient β was obtained from the slope of the stress versus strain curve using the equation $y = ae^{\beta x}$. Cumulative dose-response experiments were performed to determine vascular reactivity (Sachidanandam et al., 2006, 2008). The system was maintained at a constant pressure of 50 mm Hg at 37°C. Relaxation responses were determined using acetylcholine (Ach; 1 nM–5 μ M) and sodium nitroprusside (SNP; 0.01–100 μ M) in vessels precontracted with serotonin. Relaxation responses were calculated as a percentage change in lumen diameter from baseline, the responses being normalized to serotonin precontraction, which was taken as 100%. Dose-response curves were analyzed by curve-fitting (GraphPad Software Inc., San Diego, CA), and sensitivity EC_{50} (nanomolar) and R_{max} (percentage) values were calculated to assess sensitivity and magnitude of responses.

MMP Expression and Activity. Vascular collagenase (MMP-13) and gelatinase (MMP-2 and -9) activities were determined using fluorescein-conjugated collagen or gelatin assay kits as we described previously (Harris et al., 2005; Sachidanandam et al., 2007). In brief, snap-frozen third-order mesenteric arteries were homogenized in radioimmunoprecipitation assay buffer, and homogenates (20 μ g of

total protein) were incubated with the substrate. Increased fluorescence that is directly proportional to the proteolytic activity of MMP enzymes was measured at 24 h using a microplate fluorometer. Other serine proteases in the tissue extracts were blocked by using 50 mM phenylmethylsulfonyl fluoride. Tissue inhibitor of metalloproteinase (TIMP)-2 levels were obtained using an enzyme-linked immunosorbent assay kit (GE Healthcare, Chalfont St. Giles, UK).

Immunoblotting. Mesenteric artery homogenates were subjected to immunoblotting as described previously (Harris et al., 2005). Antibodies against MMPs (2 and 13) (Calbiochem), calponin, and α -actinin (Sigma-Aldrich) were used. Densitometric measurements were normalized using β -actin (Sigma-Aldrich) as a loading control.

Statistical Analysis. Two-way ANOVA comparing disease and diet and a post hoc Bonferroni analysis were done to determine significance between groups. A two-way ANOVA was performed to evaluate EC_{50} and R_{max} differences in vascular responses to ET-1, ACh, and SNP, comparing control and diabetic animals on normal or high-fat diet. Nonlinear regression was used to plot relaxation responses to ACh and SNP. A sigmoidal dose-response curve was plotted for 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, followed by a post hoc Bonferroni adjustment for the multiple comparisons. GraphPad Prism 5.0 was used for all analyses (GraphPad Software Inc.). Significance was considered at $p < 0.05$. All results are reported as unadjusted mean $\pm$ S.E.M.

Results

Animal Metabolics. GK rats were originally derived from selective breeding of glucose intolerant Wistar rats, which serve as the control for this model (Cheng et al., 2001; Standaert et al., 2004). They are neither hyperlipidemic nor hypertensive, allowing the opportunity to determine hyperglycemia effects in a spontaneous model of diabetes (Cheng et al., 2001; Elgebaly et al., 2008; Harris et al., 2008; Sachidanandam et al., 2008). Metabolic parameters summarized in Table 1 demonstrated that hyperglycemia was markedly augmented with the high-fat diet in the diabetic group and correlated with the long-term glucose control depicted by HbA1C levels. Weight gain during the study period was similar in both groups. The high-fat diet raised total cholesterol and low basal triglyceride levels only in diabetic animals and not in controls. Adiposity was increased in both control and diabetic groups on a high-fat diet. As previously reported by us and others, GK rats do not present with hyperinsulinemia, although they display insulin resistance as determined by euglycemic-hyperinsulinemic clamp studies (Cheng et al., 2001; Elgebaly et al., 2008). Insulin levels were increased only in combined hyperglycemia and high-fat diet. ET-1 levels in the plasma were higher in diabetic rats

and further increased upon high-fat feeding. There was no difference in MAP between the groups.

Vascular Structure. Medial thickening and narrowed lumens were observed in diabetes, thus increasing the overall M/L ratio. A high-fat diet did not further elevate the M/L ratio in diabetic animals (Fig. 1). Type 1 collagen expression was increased in diabetes or hyperlipidemia alone, and the high-fat diet did not cause a further change in diabetes (Fig. 2C). However, collagen staining patterns indicated differential distribution of mature versus new collagen in mesenteric cross sections. When given normal chow, the relative distribution of new to mature collagen was 1:5 and 5:9 in control and diabetic animals, respectively, indicating a greater increase in new collagen and thus collagen synthesis in diabetes (Fig. 2, A and B). Respective values in control and diabetic animals on the high-fat diet were 5:5 and 2:11, suggesting that in control animals, total collagen is higher because of an increase in new collagen. In contrast, in diabetic animals, there is no further increase in total collagen, but degradation appears to be lower as indicated by an increase in mature collagen.

Matrix Metalloproteinase Activity and Expression. Gelatinase activity was increased in diabetes or hyperlipidemia alone, and a high-fat diet did not cause a further increase in diabetes (Fig. 3A). There was an increase in MMP-2 protein levels with high-fat feeding in both control and diabetic animals (Fig. 3B), although MMP-9 protein levels were similar (data not shown). TIMP-2 levels were increased in diabetic animals that were given a high-fat diet (Fig. 3C). Collagenase activity was lower in diabetic animals on a regular diet. The high-fat diet did not affect MMP-13 activity in control rats, whereas in diabetic animals, there was a trend for further decrease, but it did not reach statistical significance (Fig. 3D). MMP-13 protein levels were increased in diabetic animals on either normal or high-fat diet (Fig. 3B).

Vascular Mechanics. Myogenic tone was higher in diabetic rats, and the high-fat diet reduced tone to control values (Fig. 4A). The β -coefficient, which is indicative of vessel stiffness, was elevated in both the diabetic groups compared with the control groups. There was no exacerbated effect upon addition of a high-fat diet to diabetes (Fig. 4B).

Vascular Function. Combined hyperglycemia and high-fat diet (diabetic HF group) caused hyperreactivity to ET-1, as indicated by increased R_{max} with no change in the sensitivity (EC_{50}) (Fig. 5A; Table 2). 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 have relaxation restored to baseline (Fig. 5B; Table 2). SNP was

TABLE 1
Metabolic parameters of control Wistar and diabetic GK rats on normal or high-fat diets

	Control	Control HF	Diabetic	Diabetic HF
Weight gain (g)	87.7 $\pm$ 9.3	115.2 $\pm$ 13.4*	69.8 $\pm$ 1.5	108.8 $\pm$ 8.5**
Blood glucose (mg/dl)	99.1 $\pm$ 9.4	97.6 $\pm$ 5.9	158.7 $\pm$ 12.9*	253.3 $\pm$ 32.1*.*
Total cholesterol (mg/dl)	70.5 $\pm$ 4.6	72.1 $\pm$ 5.6	88.6 $\pm$ 3.5	123.4 $\pm$ 9.2*.*
Triglycerides (mg/dl)	63.1 $\pm$ 2.6	87.0 $\pm$ 18.7	37.8 $\pm$ 0.5*	60.6 $\pm$ 8.1**
Insulin (ng/ml)	1.5 $\pm$ 0.3	1.6 $\pm$ 0.5	1.2 $\pm$ 0.2	2.1 $\pm$ 0.2**
ET-1 (pg/ml)	1.05 $\pm$ 0.07	1.98 $\pm$ 0.56*	3.5 $\pm$ 0.37*	5.75 $\pm$ 0.56*.*
HbA1C (%)	5.4 $\pm$ 0.1	5.4 $\pm$ 0.2	7.6 $\pm$ 0.6*	10.1 $\pm$ 1.0*.*
Adiposity (fat/weight gain)	0.17 $\pm$ 0.01	0.22 $\pm$ 0.01*	0.19 $\pm$ 0.01	0.28 $\pm$ 0.02*.*

* $p < 0.05$ vs. control.

** $p < 0.05$ vs. diabetic (mean $\pm$ S.E.M.; $n = 5$ –10/group).

Fig. 1. Formalin-fixed mesenteric artery cross-sections were analyzed for morphological changes by Masson staining. Diabetes caused an increase in the M/L ratio, which was not affected by high-fat diet feeding in either control or diabetic animals. Representative images are shown in A, and combined morphometric analysis is given in B. *, $p < 0.05$ compared with control; $n = 5$ to 8/group.

Fig. 2. Collagen deposition patterns were observed from picrosirius red-stained cross-sections, viewed under polarized light. There was increased new collagen in diabetes or hyperlipidemia alone compared with control (increased green/red ratio in staining). Representative images are shown in A, and combined analysis of the relative distribution of new and mature collagen is given in B. Type 1 collagen expression increased in diabetes and diet-induced hyperlipidemia alone but not in combination (C). *, $p < 0.05$ versus control normal diet; $n = 4$ to 5/group.

used to test endothelium-independent mechanisms of vasorelaxation. There was a decreased sensitivity to SNP in diabetic animals on a high-fat diet compared with the other groups. Maximal relaxation was not affected (Fig. 5C; Table 2).

Expression of Contractile Proteins. α -Actinin was up-regulated upon addition of a high-fat diet in both control and diabetic groups (Figs. 6, A and B). Calponin levels were elevated with high-fat diet treatment in diabetes alone, whereas the other groups had comparable levels (Fig. 6, A and C).

Discussion

The major findings of this study are: 1) addition of a high-fat diet does not exacerbate medial thickening and vascular compliance in type 2 diabetes; 2) high-fat diet or hyperglycemia alone cause matrix deposition, mainly because of increased collagen synthesis, whereas when combined, there is less turnover of accumulated matrix; 3) high-fat diet and diabetes combined cause impaired relaxation responses to both endothelium-dependent and -independent vasodilators, and there is an exaggerated constriction response to ET-1; and 4) there is increased expression of contractile proteins calponin and α -actinin in combined hyperglycemia and high-fat diet.

Type 2 diabetes often presents together with insulin resistance, obesity, dyslipidemia, and hypertension. Although most studies focused on changes observed in the intimal layer mediated by hyperlipidemia and diabetes, the effect of dyslipidemia on the structure and function of the contractile medial layer is not known. We used the type 2 diabetic GK rat, a lean and normotensive model, in combination with diet-induced hyperlipidemia to distinguish the effects of hyperglycemia and high-fat diet either alone or in combination (Cheng et al., 2001; Sachidanandam et al., 2006; Elgebaly et al., 2007). There have been a few reports of remodeling in models of type 1 diabetes (Cooper et al., 1994, 1997). We recently extended these studies to type 2 diabetes and showed that there is significant medial thickening in mesenteric resistance arteries in GK rats (Sachidanandam et al., 2007). We also showed that antagonism of ET_A receptors block this effect, whereas ET_B receptor blockade worsens vascular remodeling. In the current study, we confirmed that diabetes causes vascular remodeling by increasing M/L ratios, paralleled by an increase in collagen deposition as a result of increased synthesis and decreased degradation. The addition of a high-fat diet did not affect medial structure and total collagen deposition, suggesting that the remodeling pro-

Fig. 3. MMP proteins are differentially regulated in diabetes and hyperlipidemia alone or in combination. A, mesenteric artery homogenates from diabetic or high-fat diet-fed control and diabetic animals show increased gelatinolytic activity when incubated with fluorescein isothiocyanate-gelatin. There was a disease-diet interaction ($p < 0.001$) such that in the normal diet group, enzyme activity was higher in diabetes, and in the high-fat diet group, enzyme activity was greater in the control group (*, $p < 0.001$ versus control normal diet; **, $p < 0.001$ versus diabetic high-fat diet). B, MMP-2 protein levels were increased with high-fat diet treatment (*, $p < 0.001$ versus normal diet; **, $p < 0.05$ versus diabetic high-fat diet). C, TIMP-2 levels were increased in diabetic rats that received a high-fat diet (*, $p < 0.05$ versus normal diet or control high-fat diet). D, increased fluorescence that is directly proportional to the proteolytic cleavage of fluorescein isothiocyanate-collagen by MMP-13 was measured at 24 h, and it was decreased in diabetic animals given a normal or high-fat diet (*, $p < 0.001$ versus control). E, expression of MMP-13 was elevated in diabetic animals receiving either normal or high-fat diet (* $p < 0.05$ versus normal). All densitometry was normalized using β -actin as a loading control; $n = 5$ to 6/group.

Fig. 4. A, myogenic tone was assessed in the arteriograph at 60 mm Hg. Hyperglycemia increased vascular tone in diabetes. High-fat diet normalized tone to control levels in diabetes (*, $p < 0.05$ versus control normal diet or diabetic high-fat diet). B, 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 (*, $p < 0.05$ versus control); $n = 5$ to 8/group.

cess was primarily mediated by hyperglycemia. Molnar et al. (2005) reported using a mouse model of diet-induced hyperlipidemia and type 2 diabetes that although there was a worsening in cardiometabolic parameters and impaired endothelial-dependent vasodilation, there was no vascular remodeling, supporting our results.

MMPs are very important for the regulation of ECM dynamics, and these enzymes are regulated at various levels (Visse and Nagase, 2003). Increased ECM protein synthesis, diminished MMP activity, and/or increased TIMP activity all could contribute to matrix accumulation (Visse and Nagase, 2003). This study showed that there is increased collagen deposition and an up-regulation of MMP-2 expression and activity under hyperglycemic or hyperlipidemic conditions. Although original studies indicated a role for MMPs in ma-

trix degradation, recent studies suggest that MMPs, especially MMP-2 and MMP-9, are involved in vascular smooth muscle cell migration and activation of profibrotic membrane-bound proteins (Johnson et al., 2001; Shah and Catt, 2003). Thus, increased MMP-2 activity may indeed contribute to increased collagen synthesis observed in this study. When hyperglycemia and high-fat diet are combined, TIMP-2 is increased, preventing further increases in gelatinase activity and new collagen synthesis compared with the high-fat diet or diabetes alone. MMP-13 activity, which degrades fibrillar collagen, is attenuated only in diabetes and not hyperlipidemia, despite an increase in protein levels suggesting regulation at the post-translational or inhibitory checkpoints of MMP-13 activity. These results are important to demonstrate that although the high-fat diet or diabetes alone can

Fig. 5. A, vasoconstriction to ET-1 (0.1–100 nM) in resistance arteries of control Wistar and diabetic GK rats on normal or high-fat diet. Maximal constriction was increased in only diabetic rats fed the high-fat diet, and sensitivity to ET-1 remained the same across groups. *, $p < 0.05$ versus other groups; $n = 5$ to 6 per group. Endothelium-dependent and -independent vasorelaxation responses to ACh (B) and SNP (C) in control Wistar and diabetic GK rats on normal or high-fat diet. Maximal 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.” *, $p < 0.05$ versus other groups; $n = 5$ to 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.

TABLE 2

Sensitivity (EC_{50} ; nanomolar) and magnitude (R_{max} , percentage) of vascular responses to ET-1

ACh and SNP in mesenteric arteries of control Wistar and diabetic GK rats with or without HF diet administration. Data are presented as mean $\pm$ S.E.M.

	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	$74 \pm 5^*$
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	$74 \pm 6^*$
SNP				
EC_{50}	5.7 ± 2	3.5 ± 1.2	6.5 ± 2.3	$23.5 \pm 10.4^*$
R_{max}	102 ± 3	101 ± 2	99 ± 1	98 ± 2

* Interaction between disease (diabetes) and diet (high-fat diet) obtained by two-way ANOVA. $p < 0.05$; $n = 5$ to 8 per group.

promote collagen synthesis, medial thickening occurs when collagen degradation is decreased in diabetes, and the high-fat diet does not worsen this effect, at least in the 7-week diet protocol used for this study.

Mechanical properties of the vessel are interdependent of vascular structure and the components of the extracellular matrix, such as collagen, fibronectin, and elastin. Intengan and Schiffrin (1998) reported that the potent vasoconstrictor ET-1 is involved in mediating resistance artery stiffness in salt-sensitive experimental hypertension. Several other groups reported increased ET-1 and/or ET receptor expression following a high-fat diet in rodent models, and there may be differences in ET-1-mediated contractility depending on the vascular bed. Mundy et al. (2007) showed increased aortic ET_A receptor protein in high-fat-fed mice, with no change in vascular responsiveness to ET-1 (Mundy et al., 2007). Treatment with a high-fat diet did not influence ET-1-mediated contractions in the femoral artery, whereas vasoconstriction was significantly augmented in the carotid artery (Bhattacharya et al., 2008). A recent study demonstrated endothelial dysfunction and increased vascular ET-1 levels following a 4-week high-fat treatment protocol (Bourgoin et al., 2008). 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. The β -coefficient, an indicator of vessel stiffness, was higher in diabetes compared with 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. Although the high-fat diet increases total collagen in control rats, there was no change in vessel stiffness. This may be because of the fact that there is more new (thin) and not mature collagen with high-fat feeding. We believe the changes observed in stiffness are also independent of blood pressure because GK rats are normotensive, and addition of the high-fat diet does not alter the blood pressure in either group. Myogenic tone was significantly higher in diabetic animals compared with controls. Lucchesi et al. (2004) reported that MMPs 2 and 9 were both involved in myogenic tone generation via a growth factor transactivation mechanism in mice mesenteric arteries. Although we did not directly study the effect of MMP inhibition on myogenic tone in our model, the fact that we do not see an increase in myogenic tone in the high-fat group despite significant up-regulation of gelatinase activity suggests that MMPs do not contribute to vascular tone in diabetes. Interestingly, the hyperglycemia and high-fat diet combination decreased myogenic tone in the diabetic animals to match control values. Because myogenic tone is a pressure-induced response, further studies are needed to assess mesenteric blood flow in these subsets to ascertain the interaction between the two.

Before our study, the role of ET-1 in mediating vasoconstriction has been demonstrated by several groups, including our own (Miller et al., 2002; Amiri et al., 2004; Sachidanandam et al., 2006). In our current study, we could not reproduce those observations of ET-mediated hyperreactivity in diabetes, and we believe that the reason behind this variability is the difference in techniques involved to assess microvascular function. Our earlier study employed a nonperfused

Fig. 6. A, representative images for the expression of contractile proteins and densitometric analyses of α -actinin (B) and calponin (C). 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. *, $p < 0.05$ versus normal diet; **, $p < 0.05$ versus control high-fat diet; n = 5 to 6 per group.

artery preparation using the wire myograph, in which there are no interfering effects of intraluminal flow and transmural pressure. In the absence of constant flow, shear stress-induced release of NO and other endothelial vasodilators may not participate in the regulation of vascular reactivity. In addition, the vessel is tensioned to a point where it is expected to produce maximal responses to vasoactive agents, and isometric force generated is measured. However, in the arteriograph, the vessel is maintained at a constant perfusion pressure, and there is no effect of tension. Data from the arteriograph are expressed as a percentage change in lumen diameter, not considering vessel wall changes. Edvinsson et al. (2007) recently reported vascular responses in the rat middle cerebral artery using perfused and nonperfused approaches to a similar effect. With the wire-myograph, they reported over a 2-fold increase in response to vasoactive agents compared with vessels mounted on the perfused arteriograph.

ACh-induced endothelial-dependent vasorelaxation was decreased in combined diabetes and high-fat diet, consistent with results from a similar study by another group that employed a mouse model of diet-induced obesity and type 2 diabetes (Molnar et al., 2005). We also found impaired non-endothelial relaxation mediated by SNP in mesenteric microvessels of type 2 diabetic rats fed a high-fat diet, indicating smooth muscle involvement in vascular dysfunction. The cytoskeletal proteins α -actinin and calponin, which mediate vascular smooth muscle cell contraction by binding to the actin-myosin apparatus, are known to be up-regulated with hyperglycemia and hyperinsulinemia in vitro (Ha, 2006; Schaafsma et al., 2007). In our study, we observed increased expression of calponin and α -actinin in mesenteric microvessels in combined hyperglycemia and hyperlipidemia, which also displayed vascular dysfunction as hyperreactivity to ET-1 and impaired relaxation to ACh and SNP.

In summary, although vascular remodeling and impaired mechanical properties were observed in type 2 diabetes, the addition of a high-fat diet did not worsen these parameters in our experimental model. However, vascular function and not structure was impaired by the addition of a high-fat diet. These findings suggest that within the time frame studied, hyperglycemia and high-fat diet promote differential patho-

logical effects on the microvasculature; thus, it is important to target both conditions individually to prevent or treat cardiovascular disease in type 2 diabetes.

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