Evidence for Vasculoprotective Effects of ET$_B$ Receptors In Resistance Artery Remodeling In Diabetes

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Running Title: Resistance Artery Remodeling and ET-1 in diabetes

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ABSTRACT

Objective: Vascular remodeling, characterized by extracellular matrix deposition and increased media-to-lumen (M/L) ratio, contributes to the development of microvascular complications in diabetes. Matrix metalloproteinases (MMPs) play an important role in the regulation of ECM turnover and vascular remodeling. Vasoactive factor endothelin-1 (ET-1) not only causes potent vasoconstriction but also exerts profibrotic and proliferative effects that change vessel architecture, which makes it a likely candidate for a key role in vascular complications of diabetes. Thus, this study investigated the regulation of MMP activity of resistance arteries under mild-to-moderate diabetic conditions as seen in Type 2 diabetes and the relative role of ET receptors in this process.

Research Design and Methods: Vessel structure, MMP activity and ECM proteins were assessed in control Wistar and diabetic Goto-Kakizaki (GK) rats treated with vehicle, ET_A receptor antagonist Atrasentan (5 mg/kg/day) or ET_B receptor antagonist A-192621 (15 mg/kg/day) for 4 weeks.

Results: M/L ratio was increased in diabetes. Atrasentan prevented this increase whereas A-192621 caused further thickening of the medial layer. Increased MMP-2 activity in diabetes was prevented by Atrasentan treatment. Collagenase activity was significantly decreased in diabetes and while ET_A antagonism improved enzyme activity, ET_B blockade further reduced collagenase levels. Accordingly, collagen deposition was augmented in GKs which was reversed by Atrasentan but exacerbated with A-192621.

Conclusions: ET-1 contributes to the remodeling of mesenteric resistance arteries in diabetes via activation of ET_A receptors and that ET_B receptors provide vasculoprotective effects.
INTRODUCTION
Cardiovascular complications contribute to the increased morbidity and mortality in Type 2 diabetes. Pathological changes in vascular function and structure underlie these complications. Vasoactive factor endothelin-1 (ET-1) not only causes potent vasoconstriction but also exerts profibrotic and proliferative effects that change vessel architecture, which make it a likely candidate for a key role in vascular complications of diabetes. A significant correlation has been observed between plasma ET-1 levels and diabetic complications (1; 2). ETₐ receptor antagonism prevents mesenteric vascular hypertrophy in Type 1 diabetes (3). Fukuda et al. reported that blockade of ET-1 action inhibits aortic extracellular matrix (ECM) deposition (4). We showed that ET-1 levels are elevated and an ETₐ antagonist prevents ECM deposition and MMP activation in middle cerebral arteries but not in the kidney of Goto-Kakizaki (GK) rats, a non-obese Type 2 diabetes model (5; 6). ET-1 mediates its diverse effects via distinct G protein-coupled receptor subtypes, ETₐ and ETₐ. While these past studies indicate the involvement of the ETₐ receptor subtype in mediating detrimental effects of ET-1, the role of ETₐ receptors in diabetes-induced changes in the vasculature remains unknown. ETₐ receptors, localized mainly on vascular smooth muscle cells (VSMC), are responsible for the vasoconstrictile and proliferative response to ET-1 (7). Endothelial ETₐ receptors mediate vasodilatation whereas vascular smooth muscle cell (VSMC) ETₐ receptors can also lead to vasoconstriction in certain vascular beds (7). This duality of function of ETₐ receptors underscores the importance of binding and function of both ET receptor subtypes. Especially given that inhibition of the ETₐ receptor system in a knock-out mouse model or pharmacological blockade by an ETₐ antagonist leads to enhanced intimal hyperplasia observed in carotid arteries after injury induced by ligation suggesting a vasculoprotective effect (8), the question remains whether ETₐ receptors contribute to or balance detrimental effects of ETₐ receptor activation in diabetic vascular remodeling.
Vascular ECM displays a very dynamic equilibrium where there is constant synthesis, degradation and reorganization. Turnover of matrix proteins are regulated by matrix metalloproteinases (MMPs) (9). While decreased MMP activity is generally believed to contribute to ECM accumulation in diabetic kidney and in vascular tissue from patients with diabetes, we and others have recently reported that there is an early activation of MMPs in hypertension and diabetes and ET-1 is involved in regulation of MMP activity (5; 6; 10; 11). Recent reports indicated that MMPs are also important in generating myogenic tone of mesenteric arteries (12; 13). However, regulation of the MMP activity of these resistance arteries under mild-to-moderate diabetic conditions as seen in Type 2 diabetes and the relative role of ET receptors in this process remained unknown. Accordingly, this study investigated vessel structure, MMP activity and ECM proteins in control Wistar and diabetic Goto-Kakizaki rats chronically treated with vehicle, ETₐ receptor antagonist Atrasentan or ETₐ receptor antagonist A-192621. The central hypothesis was that increased MMP activity would be associated with hypertrophic remodeling of the mesenteric resistance arteries in diabetes and while ETₐ receptor blockade
would prevent this effect, antagonism of vasculoprotective \( \text{ET}_B \) receptor would display opposing effects augmenting pathological remodeling in Type 2 diabetes.

**RESEARCH DESIGN AND METHODS**

**Animal and tissue preparation.** All experiments were performed on male Wistar (Harlan, Indianapolis, IN) and GK (in-house bred, derived from the Tampa colony) rats (14; 15). The animals were housed at the Medical College of Georgia animal care facility that is approved by the American Association for Accreditation of Laboratory Animal Care. All protocols were approved by the Institutional Animal Care and Use Committee. Animals were fed standard rat chow and tap water ad-libitum until sacrifice at 18 weeks of age. Weight and blood glucose measurements were monitored twice a week till sacrifice. Blood glucose was measured from the tail vein using a commercially available glucometer (Freesytle, Alameda, CA). Blood pressure was monitored either by telemetric method (as previously reported) (6) or via the tail cuff method (Kent Scientific, Torrington, CT) which we have previously validated on telemetry implanted animals (5). After the spontaneous onset of diabetes, starting at 14 weeks of age, animals were divided into groups and treated for four weeks as follows: \( \text{ET}_A \) receptor blockade – Atrasentan (Abbott Labs) 5 mg/kg/day in drinking water, \( \text{ET}_B \) receptor blockade – A-192621 (Abbott Labs) 15 mg/kg/day by oral gavage split into two daily doses or vehicle as recommended by the manufacturer (6; 16-18). Daily water consumption was measured for Atrasentan treatment arm (6).Vehicle for the A-192621 consisted of 83% deionized water, 10% Polyethylene Glycol-400, 5% ethanol, and 2% Cremophor EL. Tap water was used as vehicle for the Atrasentan. Animals were anesthetized with sodium pentobarbital and exsanguinated via cardiac puncture. The mesenteric bed was then harvested and third order mesenteric arteries were isolated for morphometry and biochemical studies. For immunohistochemistry, mesenteric arteries were perfused with Histogel (Richard Allen Scientific, Kalamazoo, MI), then excised and embedded in the same matrix. Upon gelling of the matrix, the embedded vessel was placed in 10% formalin for storage. For protein studies, vessels were excised, snap frozen in liquid nitrogen, and stored at -80°C.

**Plasma measurements.** Plasma ET-1 and insulin were measured by specific ELISA kits from ALPCO Diagnostics (Windham, NH).

**Tissue homogenization and MMP activity.** Snap frozen mesenteric arteries were homogenized in modified RIPA buffer (50 mM Tris-HCl, 1 % Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1mM PMSF, 1 \( \mu \)g/ml aprotinin, leupeptin, pepstatin, 1 mM sodium orthovanadate, and 1 mM sodium fluoride) as we previously described (6). Gelatinolytic activity was assessed by densitometric analysis (Gel-Pro version 3.1, Media Cybernetics, Carlsbad, CA) (6). Recombinant MMP-2 and MMP-9 proteins (Calbiochem, San Diego, CA) were run in parallel with all samples and the band intensity on zymogram gels was normalized to that of standard to prevent gel-to-gel variability. Collagenase activity of vascular MMP-13 was determined using a fluorescein-conjugated collagen assay kit as recommended by the manufacturer (Molecular Probes, Eugene, Oregon). Briefly, homogenates (20 \( \mu \)g total protein) were incubated with the
substrate and increased fluorescence that is directly proportional to the proteolytic activity of MMP-13 was measured at time 0, 2, 4, 8 and 24 h using a microplate fluorometer. Other serine proteases in the tissue extracts were blocked by using 50 mM phenylmethylsulfonylfluoride (PMSF). Tissue inhibitor of metallocproteinase-2 (TIMP-2) levels were measured by enzyme-linked immunoassay (Amersham Biosciences, Piscataway, NJ).

**Vessel morphology and immunohistochemistry.** Fixed vessel segments were embedded in paraffin, sectioned at 4 microns, and mounted on treated slides. Sections were stained with Masson trichrome stain. Slides were viewed using a Zeiss Axiovert microscope (Carl Zeiss, Inc., Thornwood, NY) and media:lumen ratios (M/L) were analyzed using Spot software (Diagnostic Instruments, MI). 4 measurements were made per section and each animal had at least 3 sections. For immunohistochemistry, slides were then deparaffinized, blocked (Super Block, Biogenex Labs, Inc., San Ramon, CA), and placed in PBS for 5 minutes. Slides were then incubated with MMP-2 primary antibody at room temperature, washed, then incubated in secondary antibody (LSAB2-HRP Kit, Dako, Carpinteria, CA) followed by incubation with Streptavidin-HRP. Bound antibody was detected with DAB substrate kit. Additional slides were incubated with only the secondary antibody to determine non-specific staining.

**Immunoblotting.** Protein levels of MMP-2 was determined by immunoblotting as previously described (6; 19) and antibodies were from Calbiochem (Cambridge, MA). Collagen type 1 and collagen type 4 levels were evaluated by slot-blot analysis and antibodies were from BD Transduction Laboratories (San Jose, CA). All blots were restained with anti-actin antibody (Sigma, St. Louis, MO) for equal protein loading. Using a subset of samples, blots were hybridized with secondary antibody alone to evaluate nonspecific binding and there was no detectable signal.

**Statistical analysis.** A rank transformation was applied to the data prior to analysis to address distributional issues for all outcome variables (20). A 2x3 analysis of variance was used to investigate the main effects of Disease (control vs. diabetic) and Drug (saline vs. Atrasentan vs. A-192621 low dose) and the interaction between Disease and Drug. Effects were considered statistically significant at p<0.05. SAS® version 9.1.3 was used for all analyses.

**RESULTS**

**Metabolic parameters.** Metabolic parameters for all study groups are summarized in Table 1. Diabetic animals were significantly smaller than control and ET receptor antagonism did not affect animal weight. GK animals displayed elevated blood glucose in all treatment groups. There was a disease-drug interaction such that A-192621 treatment caused a further elevation as compared to vehicle in only GK rats but not in controls.

**Vascular structure.** GK animals exhibited increased media thickness of mesenteric arteries with a significantly increased media:lumen (M/L) ratio. ET$_A$ antagonism prevented the increase in M/L but ET$_B$ antagonism caused a further increase in M/L ratio in diabetic GK rats but not in controls. A representative image for each group is shown in Figure 1A and morphometry data from all animals studied are summarized in Figure 1B. Total collagen type 1 levels were quantified by slot-blot analysis in addition
Resistance Artery Remodeling and ET-1 in diabetes

Densitometric analysis (Fig. 2A) demonstrated increased collagen type 1 in diabetes. Furthermore, there was a disease and treatment interaction such that Atrasentan treatment attenuated collagen deposition whereas in A-192621 caused augmented accumulation in GKs (p=0.047). ETB blockade increased collagen type 1 in control rats too but it did not reach statistical significance. There was no difference in collagen type 4 levels in control vs GK animals.

**Mesenteric MMP expression and activity.** Since there is increased collagen accumulation in diabetes, first mesenteric collagenase (MMP-13) activity was assessed using a fluorogenic assay. In diabetic animals, this activity was significantly decreased (Fig. 2B). Similar to morphometry results, ETA blockade restored collagenase activity to levels seen in control animals and ETB antagonism caused a further but not significant decrease in GKs. Surprisingly A-192621 treatment significantly reduced collagenase activity whereas ETA blockade had no effect on enzyme activity in control animals. Gelatinolytic activity was evaluated using gelatin zymography, which detects MMP-2 and MMP-9-based lytic activity. A representative zymogram is shown in Fig. 3A. Lytic activity was detected mainly at 62 kDa and to a much lesser extent at 72 kDa corresponding to the active and latent forms of MMP-2, respectively. Densitometric analysis of the bands corresponding to active form demonstrated that MMP-2 activity was increased in diabetes and that ETA receptor antagonism prevented this increase in activation (Fig. 3B). ETB receptor antagonist-treated group also displayed lower activity levels as compared to vehicle treated diabetic rats. In order to determine whether increased MMP-2 activity results from an increase in protein levels, total MMP-2 protein was assessed by immunoblotting (Fig. 4A). MMP-2 levels were higher in the GKs and treatment with either antagonist had no effect on protein levels in diabetic animals. On the other hand, ETB antagonism increased MMP-2 levels in the control group. In only control and GK rats, immunohistochemistry was performed to determine localization of MMP-2 protein in the vessel wall. There was intense staining in the entire wall including both medial and adventitial layers (Fig. 4B).

Since TIMP-2 is the endogenous inhibitor of MMP-2, tissue levels were measured to determine whether the increase in MMP-2 activity arises from a decrease in its inhibitor (Fig. 4C). TIMP-2 was significantly decreased in diabetes. ETA antagonism restored TIMP-2 levels in GK rats without any effect on control animals. ETB blockade had no effect in the diabetic group and decreased TIMP-2 levels in controls.

**DISCUSSION**

This study tested the hypothesis that ETA receptor activation contributes to the remodeling of mesenteric microvessels via modulation of ECM dynamics by MMPs in Type 2 diabetes whereas ETB receptors exert a vasculoprotective effect by balancing detrimental effects of ETA receptors. This hypothesis was based on the previous studies that ET-1 stimulates collagen accumulation in aortic and mesenteric vessels in Type 1 diabetes and that pharmacological or genetic manipulation of ETB receptors results in augmented intimal remodeling in a carotid injury model (3; 4; 8; 21). Therefore, this study was designed to look at structural changes in resistance arteries of control
and diabetic animals chronically treated with an ET\textsubscript{A} or ET\textsubscript{B} receptor antagonist and also evaluate potential mechanisms of altered matrix dynamics in the microvasculature in diabetes. Our findings demonstrate for the first time that mild hyperglycemia causes medial thickening in the mesenteric arteries which is associated with increased gelatinase MMP-2 but decreased collagenase MMP-13 activity. Furthermore, MMP activation and increased media:lumen ratio can be prevented by the administration of an ET\textsubscript{A} receptor antagonist. On the other hand, ET\textsubscript{B} receptor blockade worsens the remodeling process suggesting a vasculoprotective effect of this receptor subtype.

The chemically-induced STZ model of Type 1 diabetes is most commonly used to study complications of diabetes. However, this model presents with very high glucose levels. Gilbert and colleagues reported that ET\textsubscript{A} receptor antagonism prevents mesenteric vascular hypertrophy in this model by inhibiting macrophage infiltration and epidermal growth factor signaling (3). There is also evidence from a transgenic mouse model that overexpresses ET-1 only in endothelial cells that ET-1 causes remodeling and dysfunction of mesenteric arteries but not conduit vessels like aorta (22). We have shown that treatment with an ET\textsubscript{A} antagonist prevented diabetes-induced changes in expression of MMPs and procollagen type 1 in mesenteric arteries but not in aorta (11). However, effect of ET-1 on resistance artery structure in models of Type 2 diabetes in which blood glucose levels are comparable to those seen in patients remained unknown. More importantly, relative role(s) of ET receptor subtypes in mediating vascular remodeling in diabetes were unclear. Murakoshi and colleagues reported that inhibition of the ET\textsubscript{B} receptor system in a knock-out mouse model or pharmacological blockade by an ET\textsubscript{B} antagonist led to enhanced intimal hyperplasia observed in carotid arteries after injury induced by ligation (8) suggesting that blockade of the receptor subtype may be detrimental. Thus, this study compared the effects of selective ET\textsubscript{A} versus ET\textsubscript{B} antagonism on vascular remodeling in diabetes. Our intriguing findings provide evidence that blockade of ET\textsubscript{A} receptors prevent diabetes-induced changes in vessel structure whereas ET\textsubscript{B} antagonism causes an opposing effect and causes further medial thickening of the resistance arteries. These results support a vasculoprotective role for the ET\textsubscript{B} receptor in the regulation of vessel architecture.

While diabetes has been reported to promote medial hypertrophy of mesenteric vessels characterized by collagen deposition and not VSMC hypertrophy (23), regulation of ECM dynamics in diabetes is not fully understood. MMPs are very important for ECM degradation and these enzymes are regulated at various levels (9). Increased ECM protein synthesis diminished MMP activity and/or increased TIMP activity all could contribute to matrix accumulation. To the best of our knowledge, the current study is the first to report regulation of proteins involved in ECM synthesis and degradation with respect to resistance vessel structure in Type 2 diabetes. In diabetic animals, resistance arteries display increased gelatinase MMP-2 and decreased collagenase MMP-13 activity. Since MMP-13 is responsible for degrading fibrillar collagen, attenuated degradation may be one mechanism of increased collagen deposition. Moreover,
it is becoming clear that MMPs have complex roles in the regulation of ECM. Degradation of basement membrane and internal elastic lamina by MMPs, disrupts the boundaries between vascular layers and facilitating VSMC migration (24; 25). Breakdown of fibrillar collagen reveals cryptic integrin signals buried in the ECM which serve as chemotactic stimuli for VSMC migration (25; 26). Equally important, MMPs activate membrane-bound proteins with growth-promoting properties via proteolytic cleavage (27-29). Thus, it is likely that augmented MMP-2 activity may indeed be stimulating vessel restructuring as seen in this study. An alternative explanation is that enhanced MMP activity at this stage of the disease may represent a compensatory response to prevent ECM deposition. However, whether MMPs directly contribute to this process or upregulated as a consequence of hypertrophy in the mesenteric bed remains to be determined.

Flamant et al. demonstrated that ET-1 enhances epidermal growth factor transactivation (30), a process that requires MMP activation (29). In the current study, increased MMP-2 activity was attenuated and blunted MMP-13 activity was restored by ET\textsubscript{A} receptor antagonism suggesting a dual mechanism for ET-1-mediated medial thickening in GK rats. It is also interesting to note that elevated MMP-2 activity paralleled increased MMP-2 protein levels in diabetes. However, ET\textsubscript{A} antagonism restored MMP-2 activity by not decreasing MMP-2 protein levels but rather stimulating the inhibitory TIMP-2 protein. As discussed above, ET\textsubscript{B} antagonism resulted in opposing effects as compared to ET\textsubscript{A} blockade causing a further increase in M/L ratio in diabetes. In addition, A-192621 treatment increased collagen levels and reduced collagenase activity not only in diabetic animals but also in control animals indicating a role for this receptor subtype in the regulation of collagen turnover.

Taken together, we conclude that mild hyperglycemia without confounding effects of hyperlipidemia and hypertension in the GK model of Type 2 diabetes appears to regulate MMP proteins and activity by both transcriptional and post-translational mechanisms leading to hypertrophic remodeling of resistance arteries, and ET-1 mediates these effects via activation of mainly ET\textsubscript{A} receptors. Furthermore, ET\textsubscript{B} receptors seem to balance the detrimental growth promoting effects of ET\textsubscript{A} receptor activation.

**ACKNOWLEDGMENTS**

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REFERENCES

TABLE 1. Metabolic parameters animals in treatment groups

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* p<0.0001 vs control, # p<0.01 vs GK vehicle or atrasentan, @g p<0.05 vs control groups
FIGURE LEGENDS

FIG 1. Vessel segments were analyzed for morphological changes and collagen deposition by Masson staining. Diabetes induced a two-fold increase in M/L ratio and ET_A receptor antagonism by Atrasentan prevented this increase whereas ET_B receptor blockade with A-192621 caused a further medial thickening. Representative sections are shown in Panel A and combined analysis is given in Panel B. n=5-8/group, *p<0.001 vs control, #p<0.001 vs diabetes vehicle or A-192621, †p<0.01 vs diabetes vehicle.

FIG 2. Effects of ET receptor antagonism on collagen deposition in diabetes. (A) Mesenteric collagen type I levels, assessed by slot-blot analysis demonstrated increased deposition in diabetes which was attenuated by Atrasentan but worsened by A-192621. n=5-9/group, *p<0.01 vs control vehicle, #p<0.05 vs diabetes vehicle, and †p<0.01 vs diabetes vehicle or Atrasentan. (B) MMP-13 collagenase activity was measured by incubating tissue homogenates with a fluorogenic MMP-13 substrate, which was decreased in diabetes. Similar to collagen results, Atrasentan treatment restored collagenase activity in diabetes but A-192621 reduced enzyme activity even in control animals. n=5/group, *p<0.001 vs diabetes vehicle, #p<0.001 diabetes vehicle vs diabetes Atrasentan, †p<0.001 control A-192621 vs control vehicle. Diabetes groups are indicated by broken lines.

FIG 3. MMP-2 activity is increased in diabetes. (A) Representative zymogram showing changes in vascular MMP-2 activity and (B) Densitometric analysis of lytic bands indicates an increase in MMP-2 activity that is ameliorated by both ET_A and ET_B receptor blockade. n=5-10/group, *p<0.01 vs control vehicle, #p<0.001 vs diabetes vehicle, †p<0.05 vs diabetes vehicle.

FIG 4. MMP-2 protein is increased regulated in diabetes. (A) Densitometric analysis of immunoreactive bands indicates that MMP-2 protein is increased in diabetes and while ET receptor antagonism has no effect on MMP-2 levels in diabetic group, ET_B blockade increases MMP-2 protein in control rats. n=5-10/group, *p<0.01 vs control vehicle, †p<0.05 vs control vehicle. (B) To localize increased MMP-2 protein in diabetes, frozen mesenteric artery cross-sections were immunostained with an MMP-2 antibody (n=3/group) which demonstrated diffuse staining along the vessel wall compared to control. Nonspecific staining was determined in the absence of primary antibody. (C) TIMP-2 protein is decreased in diabetes and ET_A receptor antagonism restores it to control levels whereas ET_B blockade had no effect. n=5-10/group, *p=0.05 vs control vehicle, †p<0.05 vs diabetes vehicle, ‡p<0.05 vs control vehicle.
Figure 1

A. Vehicle  Atrasentan  A-192621

Control

Diabetes

B.

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Media:Lumen ratio

**Graph:**
- Vehicle
- Atrasentan
- A-192621

* p < 0.05
# p < 0.01
Figure 2

A. Collagen type 1 (optical density)

B. Fluorescence intensity/mg protein
Figure 3

A.  

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B.  

![Bar graph](image7.png)

Gelatinolytic MMP-2 activity (optical density)

- **Vehicle**
- **Atrasantan**
- **A-192621**

Control  
Diabetes
Figure 4

A. 

MMP-2 protein (optical density)

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B. 

Control  Diabetes

C. 

TIMP-2 (ng/mg protein)

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