Effect of chronic and selective endothelin receptor antagonism on microvascular function in Type 2 diabetes

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CURRENTLY, NEARLY 21 MILLION people in the United States have diabetes, which raises their risk of experiencing heart disease and stroke by two- to fourfold. These life-threatening outcomes account for nearly 65% of all diabetes-related morbidity (1). Diabetes is increasingly described as a vascular disease, rather than merely an endocrine disorder. Thus the diabetic vasculature becomes a key component in mediating these pathological processes.

Vascular dysfunction in microvessels, described as a hyperreactivity to constrictor agents and/or a decreased relaxation to vasodilators, is associated with diabetes (32). The vascular endothelium plays a critical role in mediating basal tone, permeability, coagulation, and smooth muscle growth. It is a target for an early attack in the diabetic vascular disease process and produces vasoactive factors to maintain a delicate balance between health and disease (33, 34). Endothelin-1 (ET-1), the potent vasoconstrictor peptide, has been shown to be upregulated in diabetes in both clinical and experimental settings. It mediates its actions via its two receptors, ET types A (ET\(_\alpha\)) and B (ET\(_\beta\)). Several groups, as well as our own, have shown the presence of ET-1-mediated hyperreactivity of the microvasculature in animal models of diabetes and insulin resistance (3, 14, 15, 18, 24, 28). Amiri et al. (2) also established that endothelium-specific overexpression of ET-1 results in vascular dysfunction in the mesenteric microvessels. Katakam et al. (14) performed ex vivo contractility studies involving selective antagonists to the two ET receptor subtypes in a fructose-fed model of insulin resistance and reported an upregulation of the ET receptors and also increased ET binding (14). Given that when present on vascular smooth muscle, ET\(_\beta\) receptors behave like the ET\(_\alpha\) subtype mediating contraction whereas endothelial ET\(_\beta\) receptors promote vasorelaxation, the relative contribution of these receptors to the vascular dysfunction in Type 2 diabetes remains to be established.

Several pathways of relaxation have been elucidated in the streptozotocin (STZ) model of Type 1 diabetes, of which nitric oxide (NO) is an important mediator of vascular function (17, 20). In experimental obesity and diabetes, it has been reported that ET-1 and NO interact in regulating vascular tone via the ET\(_\alpha\) receptors (21, 27). Endothelium-derived hyperpolarizing factor (EDHF) and arachidonic acid pathways have been shown to play an important role in vascular function in insulin-resistant models (16, 22, 23). However, barring these studies, the involvement and cross talk of the ET receptors with the vasodilatory mechanisms in Type 2 diabetes are poorly understood. Thus we tested the hypotheses that ET\(_\alpha\) receptor blockade would prevent augmented vasoconstriction and improve endothelium-dependent vasorelaxation of mesenteric resistance arteries and that the antagonism of vasculo-protective ET\(_\beta\) receptors would display opposing effects in Type 2 diabetes.

RESEARCH DESIGN AND METHODS

Animals. All experiments were performed on male control Wistar (Harlan; Indianapolis, IN) and diabetic Goto-Kakizaki (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

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American Association for Accreditation of Laboratory Animal Care. All protocols were approved by the Institutional Animal Care and Use Committee. During housing, water consumption, weight, and blood glucose and pressure measurements were performed twice weekly. Glucose measurements were taken from the tail vein and measured on a commercially available glucose meter (AccuChek, Roche Diagnostics; Indianapolis, IN). Results are given as the average of the readings during the treatment period. Animals were housed in individual cages, maintained in a 12-h:12-h light-dark cycle and fed standard rat chow and tap water ad libitum, until euthanasia at 18 wk of age.

**Blood pressure monitoring.** Mean arterial blood pressure (in mmHg) was measured either by telemetry or using the tail-cuff method. The latter was validated on animals with telemetric implants, by the same tail-cuff operator each time, to yield similar results. The reproducibility of recordings with either technique was also confirmed by other researchers (12, 36). Animals on telemetry had transmitters implanted at week 12 and were allowed to recover for 2 wk. Mean arterial blood pressure was recorded from week 14 through week 18.

Tail-cuff blood pressure was measured on animals not on telemetry following the same course (4). Results are given as the average of the readings during the treatment period.

**ET receptor antagonism.** Subsets of diabetic and age-matched control rats were placed on either an ET<sub>A</sub> receptor antagonist, atrapentin (5 mg·kg<sup>-1</sup>·day<sup>-1</sup>), administered in the drinking water, or on ET<sub>B</sub> receptor antagonist, A-192621 (Abbott; Abbott Park, IL), at either 15 or 30 mg·kg<sup>-1</sup>·day<sup>-1</sup> doses, administered by oral gavage. Daily water consumption was measured for the atrapentin treatment arm. The doses were based on the recommendations of the manufacturer and from previous publications by us and others (8, 25, 35, 36). Treatment was given from week 14 through week 18, which accounted for at least 6 wk of diabetes before drug administration. The same time line and dosage were used in a recent study, in which we showed vascular remodeling in the mesenteric circulation (29). A corresponding control group was included wherein animals were given the vehicle.

**Surgical procedures.** Animals were anesthetized with pentobarbital sodium and exsanguinated via cardiac puncture. The mesenteric bed was harvested and immersed in ice-cold Krebs buffer containing (in mM) 118.3 NaCl, 25 NaHCO<sub>3</sub>, 4.7 KCl, 1.2 MgSO<sub>4</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.5 CaCl<sub>2</sub>, and 11.1 dextrose, and third-order mesenteric arteries were isolated for vascular function studies.

**Determination of vascular function.** Isometric tension exerted by the vessels was recorded via a force transducer using the wire-myograph technique (Danish Myo Technologies). The myograph chambers were filled with Krebs buffer aired with 95% O<sub>2</sub>-5% CO<sub>2</sub> (Danish Myo Technologies). The myograph technique was recorded via a force transducer using the wire- (0.1–300 nM) with or without acute ETA antagonism with BQ-123 (1 μM). Sensitivity (EC<sub>50</sub>) and maximum response (R<sub>max</sub>) values were calculated from the respective dose-response equations obtained by nonlinear regression analysis.

**Plasma measurements.** Plasma ET-1 and insulin were measured by enzyme-linked immunoassay kits (Alpco Diagnostics; Windham, NH). Plasma triglycerides and cholesterol were measured using commercial kits (Wako Diagnostics; Richmond, VA).

**ET receptor expression.** ET<sub>A</sub> and ET<sub>B</sub> receptor protein levels were determined by immunoblotting. Snap-frozen mesenteric artery segments were placed in modified radioimmunoprecipitation assay buffer (containing 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, a 1 mmol/l sodium orthovanadate, and a 1 mmol/l sodium fluoride) and sonicated at room temperature for 8–10-s bursts. Samples were placed on ice between sonications. Total protein was measured using the Bradford method (Bio-Rad, Richmond, CA). Vascular extracts (5 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% bovine serum albumin diluted in 0.2 M Tris base, 1.4 M NaCl, 0.1% Tween 20, and 0.02% NaN<sub>3</sub>. The membranes were then incubated overnight with the primary antibodies for ET<sub>A</sub> and ET<sub>B</sub> receptors as recommended by the manufacturer (Alomone; Jerusalem, Israel). The specificity of the bands was confirmed by using increasing concentrations of competing peptide for each antibody. Bands were visualized using ChemiGlow peptide for each antibody. Bands were visualized using ChemiGlow.

**RESULTS**

**Animal metabolics.** Metabolic parameters of both control and diabetic animals are presented in Table 1. Blood glucose was significantly elevated in the diabetic rats compared with controls, whereas body weight was considerably lower, similar to that seen in nonobese models of diabetes. The diabetic rats were normotensive. However, there was an increase in blood pressure with the 30 mg/kg ET<sub>B</sub> antagonist, suggesting a complete blockade of ET<sub>B</sub> receptors at this dose. Blood pressure increased starting around day 3, stabilized at day 6, and remained constant thereafter. Unexpectedly, the animals in the 30 mg/kg A-192621 group gained less weight, but there was no apparent difference in eating or activity.

**Vascular responses to ET-1.** Mesenteric arteries of diabetic rats showed hypersensitivity (EC<sub>50</sub>, 0.14 ± 0.07 vs. 1.4 ± 0.6 nm) and also increased maximal response compared with the control rats (R<sub>max</sub>, 126 ± 22% vs. 81 ± 6%; Table 2, and Fig. 1A). Upon treatment with the ET<sub>A</sub> antagonist, both control and diabetic rats showed decreased sensitivity with no significant change of maximum contractile responses (Fig. 1A and B). In control rats on 15 mg/kg A-192621, vasoconstriction was augmented over the concentration range (Fig. 1B) and there was an increase in the magnitude of constriction (200 ± 61%), suggesting an inhibition of endothelial ET<sub>B</sub>-mediated NO release that normally balances ET-1 constriction. There was a trend for decreased sensitivity to ET-1 in control rats on a 30 mg/kg dose. On the other hand, in diabetic animals, 15 mg/kg ET<sub>B</sub> receptor antagonist did not have much impact on constriction, whereas 30 mg/kg dose reduced the contractile response over the ET-1 concentration range.
with TEA resulted in a similar relaxation response (EC50, 28 vs. 27.8 nM; and Rmax, 106% vs. 103%; diabetic vs. control).

Vascular responses to ETB specific antagonists. To determine whether there is smooth muscle ETB-driven constriction in diabetes, we studied vascular responses to two selective ETB receptor agonists, sarafotoxin (S6c; EC50, 6 ± 0.8 in control vs. 5.4 ± 1 nM in diabetic rats; and Rmax, −8 ± 1% in control vs. −11 ± 2% in diabetic rats) and IRL-1620 (EC50, 2.3 ± 1.2 in control vs. 1.7 ± 0.4 nM in diabetic rats; and Rmax, −11 ± 2% in control vs. −9 ± 2% in diabetic rats) (Fig. 4). No vasoconstriction was observed with both agents, but there was relaxation. S6c was also tested in presence of BQ-123 (EC50, 4.6 ± 1.3 in control vs. 5.6 ± 1.4 nM in diabetic rats; and Rmax, −13 ± 2% in control vs. −7 ± 1% in diabetic rats), which caused further relaxation in the controls alone (P < 0.05).

ET receptor expression. To determine whether increased ET-1-mediated constriction is accompanied by changes in ET receptor density, ETα and ETβ protein levels were determined by immunoblotting of mesenteric artery homogenates (Fig. 5). There was a significant increase in ETα receptor density in diabetes, but ETβ receptor density remained unchanged.

**DISCUSSION**

It is well understood that diabetes leads to vascular dysfunction, especially in a manner involving multiple endothelial components. However, most of the studies were carried out in animal models of insulin resistance, either alone or coupled with obesity or in Type 1 diabetic models such as the STZ.
Using the Goto-Kakizaki rat, a nonobese and normotensive model of spontaneous Type 2 diabetes, we previously reported augmented responses to ET-1 and impaired relaxation to ACh in an NO-dependent manner (28). The current study sought to understand the receptor-mediated mechanisms behind these differences. The working hypothesis was that ETA antagonism would improve vascular function by attenuating constrictor responses to ET-1 and improving relaxation governed by NO, whereas ETB blockade would further exacerbate ET-1 vasoconstriction and decrease relaxor responses.

As predicted, contractile responses to ET-1 were increased in diabetic rats, and this augmentation was accompanied by increases in ET\textsubscript{A} receptor density. Atrasentan, a selective ET\textsubscript{A} antagonist, prevented this hyperreactive response. Whereas at low doses, the ET\textsubscript{B} antagonist did not further worsen vascular responses of diabetic rats to ET-1, it caused enhanced constriction in control rats. Upon the administration of a 30 mg/kg dose of ET\textsubscript{B} antagonist A-192621, vasoconstriction was markedly decreased in diabetes, contradictory to our hypothesis. Our observations could be in line with three probable explanations. First, it is possible that in diabetes, there is an increase in ET\textsubscript{B} receptor expression and a duality of action. Honore et al. (9–11) studied ET\textsubscript{B} blockade in hamsters and reported that chronic antagonism led to hypertension and increased pressor responses. However, they suggest that a heterogeneity of ET\textsubscript{B} receptor function in those located on the endothelium versus those on vascular smooth muscle cells. Endothelial ET\textsubscript{B} receptors are believed to confer protective effects by causing relax-
We did not detect a change in total ETB receptor abundance in diabetic rats. However, it should be noted that immunoblotting was done with total vessel homogenate and cannot differentiate vascular versus endothelial ETB receptors. To address this possibility using a pharmacological approach, we investigated contractile response to ETB-specific agonists S6c and IRL-1620 with the prediction that if there is an increase in smooth muscle ETB receptors, we would see a constriction rather than a dilatation conferred by endothelial ETB receptors. We observed only relaxation, and there was no difference between the groups. Studies with endothelium-denuded vessels would help clarify the contribution of endothelial versus vascular ETB receptors. Unfortunately, because of the limited availability of the ETB receptor antagonist A-192621, we examined vascular remodeling with half of the mesenteric vessels to evaluate the effect of diabetes on vascular function and structure and thus could not repeat the experiments with endothelium-denuded tissue. Interestingly, vascular structure studies showed an exacerbation of vascular remodeling in A-192621-treated (15 mg/kg) diabetic rats, indicating a vasculoprotective role for ETB receptors (29).

Another possibility is that there is ETA/ETB heterodimerization. Both homo- and heterodimerization of the two ET receptors have been reported by functional as well as fluorescence resonance studies (5, 6, 30). It is suggested that when heterodimerized, the ETA receptor overrides ETB receptor activation. Thus ETB receptor antagonism provides an ETA blockade-like effect. Harada et al. (7) reported in that in the rat anterior pituitary gland, there is ET_A and ET_B receptor het-

**Fig. 3. Vasorelaxation to ACh: role of nitric oxide (NO).** A: there was increased NO-mediated relaxation in diabetes. B: ET_A receptor antagonism at 15 mg/kg prevented, whereas ET_A receptor antagonism improved, vasorelaxation in control rats. *P < 0.05, control vs. control + A-192621 (15 mg/kg), control vs. control + atrasentan. C: impaired relaxation due to NO blockade in diabetic rats was restored with ET_A antagonism. ET_B receptor antagonist slightly worsened vasorelaxation in diabetes. Post hoc Tukey analysis: P < 0.05, diabetic + atrasentan vs. diabetic, diabetic + A-192621 (15 mg/kg), or diabetic + A-192621 (30 mg/kg). Results are shown as means ± SE; n = 4–8 rats.

**Fig. 4. Vascular responses to ETB-specific agonists.** A: sarafotoxin (S6c) induced vasodilatation in both control and diabetes. B: ET_A receptor antagonism increased vasorelaxation in control but not in diabetic rats. *P < 0.05. C: similar vasorelaxation responses to IRL-1620 were observed in control and diabetes. Results are shown as means ± SE; n = 5 rats.
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The constriction to ET-1 is completely ETA receptor dependent in an ETA-dependent manner. This phenomenon of upregulated to compensate for impaired relaxation in the compromised state in an ETA-dependent manner. This phenomenon of ET-1 and NO interaction has also been previously reported in the STZ rat model of Type 1 diabetes (17) and also in models of obesity and diabetes (21). ETA antagonism at 30 mg/kg caused a slight but significant impairment of relaxation compared with vehicle or atrasentan-treated diabetic rats. The relatively small effect of ETA receptor blockade on vascular relaxation in diabetes suggests that ETA regulates relaxation via the NO pathway only and that in the presence of L-NNA, the ETA blockade has a small effect. On the other hand, in control animals, complete ETA blockade with a 30 mg/kg dose again displays an ETA receptor blockade-like effect, whereas partial blockade with a 15 mg/kg dose worsens the relaxation. This also suggests that ETA receptors may be activating other pathways of relaxation in addition to NO and, thus, that antagonism impairs relaxation.

In light of our findings, several questions concerning vascular relaxation pathways arise. It is known that vasorelaxation in the systemic circulation, especially in resistance vessels, is mediated in a large part by EDHF and potassium channels (16, 22, 23). Our data with Goto-Kakizaki animals show that overall relaxation is not impaired due to an upregulated NO pathway contributing to almost 50% relaxation. This is consistent with reports by Amiri et al. (2) of the involvement of NO in resistance arteries of a rodent model of endothelium-specific ET-1 overexpression. Furthermore, another group has reported from clinical observations in obesity and metabolic syndrome that vascular relaxation in microvessels is largely NO dependent (13, 31). These data also suggest that there is an L-NNA-insensitive component. We were unable to block that with TEA or indomethacin. Nonetheless, further studies defining the roles of EDHF and cyclooxygenase pathways using more selective inhibitors of EDHF in diabetes are warranted.

In this study, we performed selective antagonism of the receptors to ET-1. However, the beneficial or adverse effects of selective versus dual receptor blockade and dose-dependent effects of ETA receptor blockade remain to be addressed from a therapeutic standpoint. Such studies have been performed in experimental hypertension but not in diabetes (10). Because of the vast heterogeneity between vascular beds, it is pivotal to understand these mechanisms in other circulatory systems to visualize the total picture. These studies with mesenteric vessels demonstrate that vascular dysfunction occurs and may contribute to altered regulation of vascular tone in diabetes. Whereas ETA receptor antagonism attenuates vascular dysfunction, the blockade of ETA receptors displays differential effects depending on the dose of the antagonists and the disease state. These results underscore the importance of ETA/ETB receptor balance and the interactions in modulating vascular function and their potential as a therapeutic target in diabetes. From a therapeutic standpoint, there is significant effort in developing selective ETA and dual ETA/ETB receptor antagonists that may be clinically used. However, to better understand the role of this receptor in health and disease and complement studies with genetic modulations of this receptor subtype, there is a great need for development and/or increased availability of ETA-selective antagonists in basic science research, which was a limiting factor for the current study. Results of this study can be confirmed and improved to serve as a foundation for future studies in ET biology only if pharmacological tools developed by the pharmaceutical industry are made available to the science community, even if these antagonists do not have a potential clinical use.
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