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

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Type 2 diabetes, a disease that affects more than 17 million Americans, holds a two- to sixfold increased risk for cerebrovascular disease and stroke (1,2), and 70% of patients with a recent stroke have overt diabetes or pre-diabetes characterized by impaired fasting glucose or impaired glucose tolerance (3). However, the underlying basis of this predisposition remains unclear. Diabetic vascular complications are associated with remodeling of the vessel wall in the retinal, renal, and mesenteric circulations. However, diabetes-induced structural changes in the cerebral microvessels are unknown.

The endothelium is an early target in diabetes, and dysfunction of vascular endothelial cells has a role in the diabetic vascular disease process (4). For example, the release of vasodilator and antiproliferative mediators such as nitric oxide and prostaglandin-I_2 is decreased, whereas production of endothelin-1 (ET-1) is increased (5). A significant correlation has been observed between plasma ET-1 levels and diabetes complications. In addition to being vasoconstrictive, ET-1 is also mitogenic. In streptozotocin-induced diabetes, nonselective ET receptor antagonism prevents extracellular matrix deposition in the retina as well as in the mesenteric arteries, providing evidence for a causal relationship (6,7). Nonselective blockade of ET receptors also prevents increased myogenic tone of cerebral vessels in diabetes (8), but the effect on vascular structure and the underlying mechanisms remain to be identified.

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

RESEARCH DESIGN AND METHODS
The Medical College of Georgia institutional animal care and use committee
approved all protocols. Male Wistar and Goto-Kakizaki (GK) rats were
obtained from Taconic (Germantown, NY) at 8 weeks of age. All animals were
individually housed at the Medical College of Georgia's animal care facility,
were allowed access to food and water ad libitum, and were maintained on a
12-h light/dark cycle. During housing, drinking water, weight, and blood
glucose measurements were performed twice weekly. Glucose measurements
were taken from the tail vein and measured on a commercially available
glucose meter (AccuChek; Roche Diagnostics, Indianapolis, IN). At 12 weeks
of age, when all GK animals became overtly diabetic, telemetry transmitters
were captured and analyzed using Spot software, and wall-to-lumen ratios
were displayed. Tissue inhibitor of metalloproteinase-2 (TIMP-2) levels were measured by
enzyme-linked immunosorbent assay (Amersham Biosciences, Piscataway, NJ).

Immunohistochemistry and vessel morphometry. Middle cerebral artery
segments were fixed in 10% formalin, embedded in paraffin, sectioned at 4 μm,
and mounted on treated slides. Slides were then deparaffinized, blocked
(Super Block; Biogenex Labs, San Ramon, CA), and placed in PBS for 5 min.
Slides were then incubated with ET-1 or CD68 primary antibody obtained from
Peninsula Laboratories (San Carlos, CA) and Dako (Carpinteria, CA),
respectively, at room temperature; washed; and then incubated in secondary
antibody (LSAB2-HRP kit; Dako), followed by incubation with streptavidin–
horseradish peroxidase. Bound antibody was detected with 3,3-
diaminobenzidine substrate kit. Additional slides were incubated with only the secondary
antibody to determine nonspecific staining. Slides were viewed using an
 Axiosview microscope (Zeiss, Thornwood, NY) and captured using Spot soft-
ware. For morphometric studies, 4-μm vessel segments were subjected to
Verhoeff van Gieson’s elastic staining or picro-polychrome staining. Images
were captured and analyzed using Spot software, and wall-to-lumen ratios
were calculated.

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

Statistical analysis. The profile of blood glucose changes over time was
analyzed for group differences (control versus diabetic) using a repeated-
measures ANOVA in which the interaction of group by time was the test of
interest. A Tukey adjustment was used for the post hoc comparison of the
groups at each time point. A rank transformation (17) was applied to all other
data before analysis to address issues of nonnormality. A 2 × 2 ANOVA
was used to investigate the main effects of disease (control versus diabetic)
drug (vehicle versus ABT-627) and the interaction between disease and drug.
Effects were considered statistically significant at P < 0.05. SAS version 8.2
was used for all analyses. Results are expressed as the means ± SE.

RESULTS
Animal data. Metabolic parameters for control and dia-
abetic (GK) animals are summarized in Table 1. Diabetic
animals were significantly smaller than controls, and ET(1)
antagonism did not affect animal weight. GK animals
displayed mildly elevated blood glucose and blood pres-
sure without hyperlipidemia and hyperinsulinemia. Exper-
iments were performed in diabetic and control animals to

Table 1. Metabolic parameters in control and GK rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Control + ABT-627</th>
<th>GK</th>
<th>GK + ABT-627</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.8 ± 0.04 (20)</td>
<td>0.8 ± 0.04 (20)</td>
<td>0.8 ± 0.04 (20)</td>
<td>0.8 ± 0.04 (20)</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>121 ± 4 (11)</td>
<td>121 ± 4 (11)</td>
<td>121 ± 4 (11)</td>
<td>121 ± 4 (11)</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>30 ± 8 (11)</td>
<td>30 ± 8 (11)</td>
<td>30 ± 8 (11)</td>
<td>30 ± 8 (11)</td>
</tr>
<tr>
<td>Blood pressure</td>
<td>104 ± 2 (3)</td>
<td>104 ± 2 (3)</td>
<td>104 ± 2 (3)</td>
<td>104 ± 2 (3)</td>
</tr>
</tbody>
</table>

Data are the means ± SE (n). *P = 0.001, GK vs. control; †P = 0.0001, GK vs. control; ‡P = 0.01, vehicle vs. ABT-627; §P = 0.001, vehicle vs. ABT-627.
assess glucose clearance capacity. Figure 1 indicates that the maximal response to glucose challenge was significantly higher in untreated diabetic animals versus control (259% of baseline vs. 168%, \( P < 0.05 \)). Additionally, after 30 min diabetic animals exhibited a decreased ability to clear glucose and return to normal levels, providing evidence for impaired glucose tolerance (\( P < 0.05 \)).

**Local and systemic ET-1 levels.** Plasma ET-1 levels were elevated approximately threefold in diabetic animals (\( P = 0.001 \)) (Fig. 2A). Tissue ET-1 was assessed by immunohistochemistry and, as shown in Fig. 2B, there was enhanced endothelial and adventitial staining in the middle cerebral arteries from diabetic animals compared with controls. Staining in the absence of the primary ET-1 antibody was determined to eliminate nonspecific staining by the secondary antibody.

**Cerebrovascular MMP expression and activity.** MMP activity in middle cerebral arteries from treated and untreated diabetic GK and control Wistar rats was assessed using gelatin zymography. A representative zymogram is shown in Fig. 3A. Lytic activity, corresponding with the molecular weight of active MMP-2, was detected in all samples. Densitometric analysis demonstrated that MMP-2 activity was increased in diabetes (\( P < 0.0001 \)) and that ETA receptor antagonism prevented this increase in activation (\( P = 0.001 \) vs. untreated GK) (Fig. 3A). Because...
TIMP-2 is the endogenous inhibitor of MMP-2. TIMP-2 levels were measured to determine whether the increase in MMP-2 activity arises from a decrease in its inhibitor. Although there was no significant difference between the control and diabetic groups, there was a trend for increased TIMP-2 levels with ETA antagonism.

To determine whether increased MMP-2 activity is caused by an increase in protein levels, total MMP-2 protein in middle cerebral arteries was assessed by immunoblotting. Two bands of 72 and 67 kDa were detected corresponding to the zymogen and cleaved active forms, respectively. A representative immunoblot is shown in Fig. 4A. Densitometric analysis of both bands indicated a fourfold increase in MMP-2 protein in diabetes (P < 0.0001 vs. control). However, unlike the zymography studies, ETA receptor antagonism did not cause a change in MMP-2 protein levels (Fig. 4A). Because MMP-1 is the major MMP that degrades fibrillar collagen, protein levels were determined in middle cerebral arteries by immunoblotting. There was a significant decrease in MMP-1 levels in GK rats (P = 0.003) and a trend toward restoration of MMP-1 levels to control values with ETA receptor antagonism (Fig. 4B).

**Vascular structure.** Verhoeff van Gieson elastic staining was performed to determine vessel diameter and wall thickness (Fig. 5A). There was a twofold increase in the wall-to-lumen ratio in GK rats that was significantly reduced by ETA receptor antagonism (P = 0.042) (Fig. 5B). Compared with control rats, there was increased staining for smooth muscle fibers and collagen, as evidenced by deep purple staining by Verhoeff van Gieson elastic staining in the cross-sections from GK rats. These changes were detected primarily in the medial layer. To further evaluate the collagen deposition dynamics, picro-polychrome staining was used to differentiate the old and newly laid collagen. In untreated GK rats, there was increased blue staining for new collagen, masking the red staining for old collagen, indicating increased collagen synthesis. ETA antagonism reduced the staining pattern for new collagen.

To determine whether and to what extent inflammation contributes to the increased wall thickness and collagen deposition, the same vascular cross-sections were immunostained with CD68 antibody, a marker for macrophages. There was no difference between groups (data not shown).

**DISCUSSION**

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

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

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

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

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

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