Endothelin antagonism prevents early EGFR transactivation but not increased matrix metalloproteinase activity in diabetes

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Endothelin antagonism prevents early EGFR transactivation but not increased matrix metalloproteinase activity in diabetes. Am J Physiol Regul Integr Comp Physiol 290: R435–R441, 2006. First published October 20, 2005; doi:10.1152/ajpregu.00300.2005.—Although past studies have demonstrated decreased renal matrix metalloproteinase (MMP) activity in type 1 diabetes and in mesangial cells grown under high glucose conditions, renal MMP expression and activity in type 2 diabetes and the regulation of MMPs by profibrotic factors involved in diabetic renal complications such as endothelin-1 (ET-1) remained unknown. The renal expression and activity of MMPs in type 2 diabetic Goto-Kakizaki (GK) rats treated with vehicle or ET_A receptor selective antagonist ABT-627 for 4 wk were assessed by gelatin zymography, fluorogenic gelatinase assay, and immunoblotting. In addition, expression and phosphorylation of epidermal growth factor receptor (EGFR) and connective tissue growth factor were evaluated by immunoblotting. Renal sections stained with Masson trichrome were used to investigate kidney structure. MMP-2 activity and protein levels were significantly increased in both cortical and medullary regions in the GK rats. Membrane-bound MMP (MT1-MMP), MMP-9, and fibronectin levels were also increased, and ABT-627 treatment did not have an effect on MMP activity and expression. Histological analysis of kidneys did not reveal any structural changes. Phosphorylation of EGFR was significantly increased in the diabetic animals, and ABT-627 treatment prevented this increase, suggesting ET-1-mediated transactivation of EGFR. These results suggest that there is early upregulation of renal MMPs in the absence of any kidney damage. Although the ET_A receptor subtype is not involved in the early activation of MMPs in type 2 diabetes, ET-1 contributes to transactivation of growth-promoting and profibrotic EGFR.

APPROXIMATELY 17 MILLION PATIENTS suffer from type 2 diabetes in the United States alone, and nephropathy resulting from microvascular complications contributes to the increased morbidity and mortality in diabetes (2). Both experimental and clinical studies demonstrated that hyperglycemia-induced changes, including mesangial and vascular smooth muscle cell growth, basement membrane thickening, as well as extracellular matrix (ECM) deposition, play an important role in the pathophysiology of kidney disease in diabetes (1, 4, 5, 28, 37). ECM has a rapid turnover because of the constant synthesis and degradation of ECM proteins by matrix metalloproteinases (MMP). This rapid synthesis and degradation process is tightly controlled by tissue inhibitors of metalloproteinases (TIMPs) (26, 43). The combination of increased ECM protein synthesis, diminished MMP activity, and/or increased TIMP activity could contribute to matrix accumulation in diabetes (15). Interestingly, recent studies demonstrated that MMPs also contribute to vascular smooth muscle cell (VSMC) growth and migration, as well as increased collagen synthesis (11, 33). Hyperglycemia or experimental type 1 diabetes decreases MMP-2 and membrane type 1 (MT1)-MMP activity in mesangial cells, which has been proposed to lead to mesangium expansion observed in diabetic nephropathy (21, 23, 38, 39). We have shown that vascular expression and activity of MMP-1, MMP-2, and MT1-MMP, as well as an MMP inducer protein (EMMPRIN) is decreased in diabetic patients (29). However, the early effect of mild hyperglycemia reflective of blood glucose levels observed in patients with type 2 diabetes on renal MMP expression and activity remains unknown.

Previous studies have shown that endothelin-1 (ET-1) plays an important role in the pathophysiology of diabetic nephropathy (8, 17). In addition to acting as a vasoconstrictor with subsequent renal hyperperfusion, ET-1 also acts a growth factor causing increased collagen and fibronectin synthesis and deposition in type 1 diabetes (6). Treatment with a dual ET receptor antagonist bosentan, as well as with an ET_A-selective antagonist LU 135252 normalized the renal matrix protein expression and prevented the development of proteinuria and renal injury (14). ET-1 inhibits MMP-2 and MT1-MMP expression in mesangial cells (46). Whether ET-1 contributes to increased matrix deposition via stimulation of matrix protein synthesis and/or via the regulation of renal MMP proteins resulting in decreasing matrix degradation in type 2 diabetes has not been studied.

Hypertrophic processes associated with diabetic nephropathy may require the interaction of various growth factors. Recent studies implicated that angiotensin (ANG) II and ET-1, both of which contribute to renal dysfunction, stimulate collagen expression via transactivation of epidermal growth factor receptors (EGFR) (11, 33). To this end, the role of ET-1 on renal EGFR transactivation in type 2 diabetes has yet to be explored. Connective tissue growth factor (CTGF) has been suggested to be an early predictor of diabetic nephropathy (24, 31, 32). Building on these past studies, we hypothesized that the expression and activity of cortical and medullary MMPs in Goto-Kakizaki (GK) rats, a nonobese and spontaneous model of mild type 2 diabetes, is altered, and treatment with selective
ET\textsubscript{A} receptor antagonist restores diabetes-induced changes in MMP activity. The effect of ET-1 on renal ECM protein expression, as well as on potential mediators, EGFR transactivation and CTGF expression, was also studied.

**MATERIALS AND METHODS**

_Needs_. Animal care and experiments were conducted in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996) and approved by the Institutional Animal Care and Use Committee of Medical College of Georgia. Male Wistar and GK rats were obtained from Taconic, (Germantown, NY) at 8 wk of age. All animals were housed at the Medical College of Georgia’s animal care facility, allowed access to food and water ad libitum, and maintained on a 12:12-h light-dark cycle. During housing, drinking water measurements, weight, and blood glucose measurements were performed twice a week. At 12 wk of age, when all GK animals became diabetic, telemetry transmitters were implanted for blood pressure monitoring. Treatment was maintained until the time of death at 18 wk of age, and blood pressure was monitored by telemetry transmitters were implanted for blood pressure monitoring, as we have reported previously (45). After a 2-wk-recovery period, control and diabetic animals were placed on the ET\textsubscript{A} antagonist ABT-627 (5 mg kg\textsuperscript{-1} day\textsuperscript{-1}) or placebo (9). The drug was dissolved in drinking water at a concentration based on the animal’s weight and daily water consumption. Treatment was maintained until the time of death at 18 wk of age, and blood pressure was monitored by telemetry throughout the treatment period. Animals were anesthetized with pentobarbital sodium and exsanguinated via the abdominal aorta. Blood was collected, and plasma samples were separated immediately and frozen in microcentrifuge tubes containing 0.005% butylated hydroxytoluene to prevent oxidation. Kidneys were immediately removed, and one kidney was fixed in formalin for morphological studies. Medulla and cortex regions of the second kidney were dissected and frozen separately in liquid nitrogen for MMP activity and immunoblotting experiments. All groups included at least 5 animals per group unless otherwise indicated.

**Measurement of metabolic parameters.** Blood glucose was measured from the tail vein after 3–4 h fasting using Accucheck glucometer (Roche Diagnostics, Alameda, CA). Serum cholesterol and triglycerides were determined using colorimetric assay kits (Wako Diagnostics, Richmond, VA). Plasma insulin was assayed with a rat insulin enzyme immunoassay kit from Alpco (Windham, NH). ET-1 levels were measured using an ELISA kit from (American Research Products, Belmont, MA), as we reported previously (10). Urinary microalbumin and creatinine levels were measured using kits from Exocell (Philadelphia, PA) and Sigma (St. Louis, MO), respectively.

**Real-time PCR.** The real-time PCR was performed in a SmartCycler II (Cepheid, Sunnyvale, CA) by using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Primer sequences for collagen type I and IV alpha 1 chains were based on the sequence information of GenBank database. 2–2.5 \mu l of cDNA template was used for the real-time PCR in a final volume of 25 \mu l. cDNA was amplified according to the following condition: 95°C for 15 s and 60°C for 60 s from 40 to 45 amplification cycles. Fluorescence changes were monitored with SYBR Green every cycle. Melting curve analysis was performed (0.5°C/s increase from 55–95°C with continuous fluorescence readings) at the end of cycles to ensure that single PCR products were obtained. Amplicon size and reaction specificity were confirmed by 2.5% agarose gel electrophoresis. All reactions were performed in triplicate. Results were evaluated with the SmartCycler II software. GAPDH primers were used to normalize samples.

**MMP activity.** MMP activity in the medulla and cortex was determined with gelatin zymography, as well as with a fluorogenic gelatinase assay. Zymography was carried out, as we previously reported (9, 29). Gelatinase activities were determined using a fluorescein-conjugated gelatin assay kit. Briefly, extracts (20 \mu g total protein) from control and GK rats (n = 5 in each group) were incubated with the substrate and increased fluorescence that is directly proportional to the proteolytic activity of MMP-2 and MMP-9 was measured with gelatin zymography.

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**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>Blood glucose, mg/dl*</td>
<td>108±3</td>
<td>113±4</td>
<td>168±5</td>
<td>168±21</td>
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<tr>
<td>Plasma insulin, ng/ml†</td>
<td>2.3±0.2</td>
<td>2.1±0.3</td>
<td>1.5±0.2</td>
<td>0.7±0.1</td>
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<tr>
<td>Plasma total cholesterol, mg/dl</td>
<td>121±4</td>
<td>124±7</td>
<td>139±11</td>
<td>125±5</td>
</tr>
<tr>
<td>Plasma triglycerides, mg/dl*</td>
<td>39±8</td>
<td>46±6</td>
<td>25±4</td>
<td>14±2</td>
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<tr>
<td>ET-1, fmol/ml*</td>
<td>0.3±0.04</td>
<td>0.3±0.07</td>
<td>0.8±0.1</td>
<td>0.5±0.06</td>
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<tr>
<td>Body weight, mg*</td>
<td>564±41</td>
<td>607±11</td>
<td>411±39</td>
<td>409±29</td>
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<tr>
<td>Mean arterial blood pressure, mm-Hg†</td>
<td>104±2</td>
<td>98±5</td>
<td>121±1</td>
<td>112±1</td>
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<tr>
<td>Urine volume, ml/day</td>
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<td>15±2</td>
<td>16±1</td>
<td>17±1</td>
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<tr>
<td>Urinary microalbumin, mg/day</td>
<td>5±0.5</td>
<td>7±4.6</td>
<td>8±0.4</td>
<td>9±1.4</td>
</tr>
<tr>
<td>Urinary creatinine, mg/day*</td>
<td>2.3±0.5</td>
<td>1.7±0.1</td>
<td>1.3±0.03</td>
<td>1.5±0.1</td>
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Values are presented as means ± SE. \*P < 0.05 Goto-Kakizaki (GK) vs. control; †P < saline vs. ABT-627.

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Fig. 1. Total matrix metalloproteinase-2 (MMP-2) activity is increased in diabetes. A: a representative zymogram showing changes in renal MMP-2 and pro-MMP2 activity. Recombinant MMP-2 standard is indicated by the arrows. B: combined densitometric analysis of lytic bands corresponding to MMP-2 and pro-MMP2 in all of the samples analyzed is shown by the bar graph and indicates an increase in total medullary MMP-2 activity that is not ameliorated by the endothelin A (ET\textsubscript{A}) receptor blockade. C: control; GK, Goto-Kakizaki. Values are presented as means ± SE; n = 8 per group, \*P < 0.05 vs. control.
measured at time 0, 15, 45, and 180 min using a microplate fluorometer. The activity of samples at 45 min, when the fluorescence intensity was at a linear range with increasing concentrations of recombinant MMP-2, was reported as fluorescence per milligrams protein. The activity of samples at 45 min, when the fluorescence intensity was at a linear range with increasing concentrations of recombinant MMP-2, was reported as fluorescence per milligrams protein per minute.

Western blot analysis. Protein levels of MMP-2, MMP-9, fibronectin, EGFR, and CTGF were determined by immunoblotting, as we previously described (9, 29). Bands were visualized using ECL detection kit from Amersham Life Sciences (Arlington Heights, IL). Antibodies for MMP-2, MMP-9, and EGFR (native and phosphorylation-specific) were obtained from Calbiochem (Cambridge, MA), antibodies for fibronectin were obtained from Chemicon (Temecula, CA); antibodies for Hb-EGF was obtained from R&D Systems (Minneapolis, MN), and antibodies for CTGF were obtained from ABCAM (Cambridge, MA). A positive control for phosphorylated EGFR that is supplied by the company was included in all immunoblots. All membranes were stripped and rebotted with an antiactin antibody to ensure equal protein loading, and densitometric analyses of the immunoreactive bands were normalized to actin levels.

Assessment of renal histology and matrix deposition. Kidneys fixed in 10% formalin were embedded in paraffin, sectioned at 4-μm thickness, and mounted on slides, which were then stained with Masson trichrome staining technique to visualize collagen content, which stains blue as opposed to black nuclei and red background. Images were captured using Axiovert-inverted microscope and SPOT software. Cortex or medullary sections were randomly selected, and collagen staining intensity was determined qualitatively.

Statistical analysis. A rank transformation was applied to the data before analysis to address issues of nonnormality (3). A 2 × 2 ANOVA was used to investigate the main effects of disease (control vs. diabetic) and drug (saline vs. ABT-627) and the interaction between disease and drug. Results are given as means ± SE. Effects were considered statistically significant at P < 0.05. SAS version 8.2 was used for all analyses.

RESULTS

Metabolic profile of animals. GK rats developed hyperglycemia around week 12 at the time the telemetry transmitters were implanted and at the end of the study; the blood glucose averaged 168 ± 5 (n = 19) vs. 108 ± 3 (n = 17) mg/dl and treatment with ETA antagonist ABT-627 (n = 10 per control or GK) did not have an effect on blood glucose (Table 1). Plasma insulin levels were significantly lower in the GK group (n = 13) than control (n = 11) and ABT-627 (n = 10 per group) further lowered the plasma insulin in both control and Geks. There was no difference in plasma cholesterol between the groups. Blood pressure was mildly elevated in the GK rats, and ABT-627 lowered blood pressure in both control and GK rats. Circulating ET-1 levels were higher in diabetic animals (n = 13) than in controls (n = 20). There was no statistically significant difference in urinary microalbumin (mg/day) levels, but creatinine (mg/day) was decreased in the GK rats.

Renal MMP expression and activity. MMP-2 and -9-dependent gelatinase activity, which has been reported to be downregulated in diabetes, was assessed by gelatin zymography in the renal cortex and medulla separately. There was very faint MMP-9 activity with no difference between the groups (not shown). Two gelatinolytic bands at 72 and 62 kDa, corresponding to proMMP-2 and MMP-2, respectively, were detected in all specimens, and total activity, corresponding to both of these

<table>
<thead>
<tr>
<th></th>
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<th>Medulla</th>
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<tr>
<td>C</td>
<td>+ ABT-627</td>
<td>+ ABT-627</td>
</tr>
<tr>
<td>GK</td>
<td>+ ABT-627</td>
<td>+ ABT-627</td>
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Values are presented as means ± SE. Total renal gelatinase activity was measured (in fluorescence·mg protein⁻¹·min⁻¹). *P < 0.05 vs. C or C + ABT-627; n = 5 per group. C, control rats.

Fig. 2. A representative immunoblot demonstrating the protein expression levels of MMP-2 (A) and MMP-9 (B) in renal tissue. Immunoreactive bands corresponding to the molecular weight of MMP-2 (62 kDa) and MMP-9 (82 kDa) were detected. Densitometric analysis of immunoreactive bands in all the samples analyzed indicates that both MMP proteins are increased in the medulla and cortex of diabetic GK rats, and ETA antagonism does not prevent this increase. Values are presented as means ± SE; n = 5 per group, *P < 0.05 vs. control.
isoforms, was significantly elevated in the medulla and cortex of GK rats compared with controls (Fig. 1). ABT-627 treatment did not have an effect on MMP-2 activity in any of the groups. To confirm zymography results, a fluorescence-based gelatinase activity assay that measures total MMP-2 and MMP-9-dependent activity was performed (Table 2). A significant increase in gelatinase activity in both cortical and medullary regions in the GKS was observed, and ABT-627 treatment failed to decrease gelatinase activity.

To investigate whether the increased MMP activity is associated with changes in protein levels, immunoblotting experiments were performed. As evident from Fig. 2A, MMP-2 protein expression was significantly higher in the GKS, and ET<sub>A</sub> antagonism did not have an effect. Although MMP-9 activity was faint in the zymograms, MMP-9 protein was detected in all the groups, and it was significantly elevated in the diabetic rats (Fig. 2B). MT1-MMP, which activates proMMP-2 to MMP-2, was also increased in the medulla of the GKS (Fig. 3A). In the cortical homogenates, MT1-MMP was increased in both ABT-627-treated controls, as well as in untreated GKS (P = 0.04). ECM protein fibronectin was significantly higher in the diabetic animals (Fig. 3B).

**Renal EGFR phosphorylation.** To determine whether and to what extent ET-1 contributes to renal EGFR transactivation in diabetes, native and phosphorylated EGFR levels were determined by immunoblotting. As shown in Fig. 4, there was no
difference in EGFR levels between control and diabetic GK rats in the medulla (P = 0.06), but in the cortex, EGFR protein was higher in the GK animals (P = 0.02). The phosphorylated form of EGFR was significantly higher in the GKS, and ABT-627 treatment reduced EGFR phosphorylation in GK rats more than in controls. The ratio of phosphorylated EGFR to EGFR was also higher in the diabetic GKS than in controls (Fig. 4B). In addition, there was a small, but significant, increase in Hb-EGF protein levels in the cortex of diabetic rats, and ABT-627 treatment prevented this increase (Fig. 4C). CTGF expression, which is considered to be an early marker of kidney damage, was assessed in the urine, as well as in renal homogenates by immunoblotting. There were low levels of expression in both specimens, which was similar in control and diabetic animals (data not shown).

Renal histology. Kidney sections were stained by Masson trichrome staining to evaluate the kidney structure. There were no apparent morphological changes or collagen accumulation in the GK rats compared with controls, as indicated by blue staining around the glomeruli or tubulointerstitial area (Fig. 5A). Collagen type I and IV alpha I gene expression was assessed by real-time PCR which demonstrated a two- and threefold increase, respectively, in mRNA levels in the cortex of GK animals compared with control animals. In ABT-627-treated animals, the expression was similar to control animals, indicating that ETA receptor antagonism prevented this increase in the diabetic rats (Fig. 5B).

**DISCUSSION**

Past studies reported that hyperglycemia causes matrix accumulation as a result of increased ECM synthesis and decreased degradation via downregulation of MMPs in streptozotin (STZ) model of type 1 diabetes, as well as in cell culture models. It is also known that profibrotic ET-1, chronically elevated in both type 1 and type 2 diabetes, contributes to renal complications. Accordingly, the overall goal of this study was to determine the regulation of renal MMPs in type 2 diabetes and whether and to what extent ET-1 contributes to this process. The important findings are several-fold. First, mild elevations in blood glucose comparable to levels seen in type 2 diabetic patients increase renal MMP-2 activity, as well as MMP-9 and MT1-MMP levels, as early as 6 wk after the onset of diabetes before there are any changes in renal structure and function. Second, ETA receptor antagonism does not prevent the early stimulation of MMPs. Finally, there is increased phosphorylation of the EGFR in the diabetic animals, and ETA receptor antagonism prevents this increase, providing evidence that ET-1 transactivates the EGFR in the diabetic kidney.

Several laboratories reported that hyperglycemia decreases MMP activity in mesangial cells (20, 22–24, 38). In these studies, cultured cells were used and exposed to high glucose (25 mM) concentrations for 5 days. Both MMP-2 activity and protein levels were significantly decreased compared with cells grown in media containing 5 mM glucose. One study, however, reported increased mRNA levels of MMPs under high-glucose conditions. Subsequent studies demonstrated that renal MMP activity was decreased in STZ-diabetic rats after 1 (39) or 6 (21) mo of diabetes. To the best of our knowledge, this is the first report on renal MMP expression and activity in type 2 diabetes. Our results demonstrate that mild hyperglycemia for 6 wk stimulates MMP-2 activity and related MMP proteins such as MMP-9 and MT1-MMP, both of which can cleave proMMP-2 to generate MMP-2. As discussed above, Song et al. (39) reported that 1 mo of STZ-induced diabetes decreases MMP activity. The reason for this difference from our results may be due to the model used. In STZ-induced diabetes, blood glucose levels are relatively high, whereas the GK model is mildly diabetic. The functional significance of increased MMP activity is also not fully understood. Although MMPs are mainly known for their matrix-degrading capacity, this family of proteases can also activate growth factors with profibrotic properties (11, 33, 42). Thus one possible explanation is that there is temporal regulation of MMPs with an early activation that triggers increased collagen synthesis and a later downregulation to promote decreased degradation. Our data demonstrate...
that there is about threefold upregulation of collagen type I and IV alpha I mRNA in diabetic kidney. Alternatively, increased MMP activity may be a compensatory response to prevent ECM deposition at the time point used in this study.

It is not surprising that we did not observe any changes in the renal structure in the GK model. Several groups reported that this model does not develop any kidney damage until 30 wk of diabetes and thus is a good model to study slow developing kidney disease as seen in most of the type 2 diabetic patients (18, 34, 35). In the current study, there was stimulation of the MMP system in the absence of any kidney damage or structural changes. In this model there is a mild elevation of blood pressure. However, we believe that changes in MMP activity and expression were independent of blood pressure as ET receptor antagonism significantly lowered the blood pressure without any change in MMP expression and activity.

There are a number of studies that demonstrated that blockade of the ET system ameliorates glomerular injury in animal models of diabetic nephropathy (6, 7, 14, 16, 40, 41). With the exception of one study in Otsuka Long-Evans Tokushima Fatty rats (41), almost all of these studies were conducted with STZ-induced diabetic animals with blood glucose levels above 400 mg/dl. These past studies reported that either ETα-selective or nonselective dual inhibition of ETα and ETβ receptors decrease glomerular injury, microalbuminuria, mesangium expansion, and fibrosis. We have found that plasma ET-1 levels decrease glomerular injury, microalbuminuria, mesangium expansion and fibrosis. In this model there is a mild elevation of blood pressure. However, we believe that changes in MMP activity and expression were independent of blood pressure as ET receptor antagonism significantly lowered the blood pressure without any change in MMP expression and activity.

ACKNOWLEDGMENTS

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