Endothelin Antagonism Prevents Early EGFR Transactivation But Not Increased Matrix Metalloproteinase Activity in Diabetes

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Summary

While past studies demonstrated decreased renal MMP activity in Type 1 diabetes and in mesangial cells grown under high glucose conditions, renal MMP expression/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/activity of MMPs in Type 2 diabetic Goto-Kakizaki (GK) rats treated with vehicle or ET\textsubscript{A} receptor selective antagonist ABT-627 for 4 weeks were assessed by gelatin zymography, fluorogenic gelatinase assay and immunoblotting. In addition, expression/phosphorylation of epidermal growth factor receptor (EGFR) and connective tissue growth factor was 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. A 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/expression. Histological analysis of kidneys did not reveal any structural changes. Phosphorylation of epidermal growth factor receptor (EGFR\textsuperscript{P}) 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 up-regulation of renal MMPs in the absence of any kidney damage. While the ET\textsubscript{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.
INTRODUCTION

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 and 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 due to constant synthesis and degradation of ECM proteins by matrix metalloproteinases (MMP). This rapid synthesis and degradation process is tightly controlled by tissue inhibitors of metalloproteases (TIMPs) (26, 43). The combination of increased ECM protein synthesis, diminished MMP activity and/or increased TIMP activity all 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, 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 hypoperfusion, 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 decreased 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 is 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 non-obese and spontaneous model of mild Type 2 diabetes, is altered and treatment with selective ET$_A$ 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.
RESEARCH DESIGN AND METHODS

Animals. Animal care and experiments were conducted in accordance with the National Institutes of Health "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, Inc. (Germantown, NY, USA) at 8 weeks of age. All animals were 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 12/12 hour light/dark cycle. During housing, drinking water measurements, weight, and blood glucose measurements were performed twice a week. At 12 weeks of age, when all GK animals became diabetic, telemetry transmitters were implanted for blood pressure monitoring as we previously reported (45). After a 2 week-recovery period, control and diabetic animals were placed on the ET\textsubscript{A} antagonist ABT-627 (5 mg/kg/day) 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 sacrifice at 18 weeks of age and blood pressure was monitored by telemetry throughout the treatment period. Animals were anesthetized with sodium pentobarbital and exsanguinated via the abdominal aorta. Blood was collected and plasma samples separated immediately were frozen in microcentrifuge tubes containing 0.005% butylated hydroxytoluene (BHT) 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 Accuchek glucometer (Roche Diagnostics). Serum cholesterol and triglycerides were determined using colorimetric assay kits (Wako Diagnostics, Richmond, VA). Plasma insulin was assayed with a rat insulin EIA 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 assessed using kits from Exocell, Inc (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, USA). Primer sequences for collagen type I and IV alpha I chains were based on the sequence information of GeneBank database. 2-2.5 µl of cDNA template was used for the real-time PCR in a final volume of 25 µl. 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 after 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. Glyceraldehyde-3-phosphate dehydrogenase (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 µg 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 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/mg protein.min.

**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 Height, IL). Antibodies for MMP-2, MMP-9 and EGFR (native and phosphorylation-specific) were from Calbiochem (Cambridge, MA), for fibronectin from Chemicon (Temecula, CA), Hb-EGF from R & D System, Inc. (Minneapolis, MN) and for CTGF 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 re-blotted with an anti-actin 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 micron thickness and mounted on slides, which were then stained with Masson trichrome staining technique to visualise
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 prior to analysis to address issues of non-normality (3). A 2x2 analysis of variance 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 mean ± SEM. 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 GKS. There was no difference in plasma cholesterol between the groups. Blood pressure was mildly elevated in the GKS and ABT-627 lowered blood pressure in both control and GKS. 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 GKS.
**Renal MMP expression and activity.** MMP2- and -9-dependent gelatinase activity, which has been reported to be down-regulated 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 isoforms were significantly elevated in the medulla and cortex of GK rats as compared to controls (Fig. 1). ABT-627 treatment did not have an effect on MMP-2 activity in any of the groups. In order 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 Figure 2A, MMP-2 protein expression was significantly higher in the GKs and ET\textsubscript{A} 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.** In order 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 EGFR-P to EGFR was higher (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 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 as compared to 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 2 and
3-fold increase, respectively, in mRNA levels in the cortex of GK animals as compared
to control animals. In ABT-627 treated animals, the expression was similar to control
animals, indicating that ET_A 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 down-regulation of MMPs in streptozosin 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 weeks after the onset of diabetes before there are any changes in renal structure and function. Second, ET\textsubscript{A} receptor antagonism does not prevent the early stimulation of MMPs. Finally, there is increased phosphorylation of the EGFR in the diabetic animals and ET\textsubscript{A} 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 as compared to 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) months 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 weeks 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 reported that 1-month of STZ-induced diabetes decreases MMP activity (39). The reason for this difference from our results may be due to the model used. In STZ-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. While 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 down-regulation to promote decreased degradation. Our data demonstrate that there is about 3-fold up-regulation 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 till 30 weeks 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/expression were independent of
blood pressure since ET receptor antagonism significantly lowered the blood pressure without any change in MMP expression/activity.

There are 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 (OLEFT) rats (41), almost of these studies were conducted with STZ-diabetic animals with blood glucose levels above 400 mg/dl. These past studies reported that either ET_{A}-selective or nonselective dual inhibition of ET_{A} and ET_{B} receptors decrease glomerular injury, microalbuminuria, mesangium expansion and fibrosis. We have found that plasma ET-1 levels are significantly elevated in this early stage of diabetes but ET_{A} receptor antagonism with ABT-627 does not prevent the activation of MMPs in the diabetic GK rats. However, ABT-627 treatment significantly reduced the EGFR transactivation as evidenced by decreased phosphorylation of EGFR without any changes in the EGFR protein levels. EGF and its receptors may contribute to diabetic kidney disease via several mechanisms. Enlargement of the kidney occurs in diabetes (13, 36) and EGF has been postulated to contribute to stimulate tubular cell proliferation (27, 44). In addition to directly stimulating growth, EGF also inhibits apoptosis (12, 19). A recent study demonstrated that EGFR inhibition attenuates early kidney enlargement in experimental diabetes via inhibition of tubular epithelial cell proliferation and stimulation of apoptosis (44). Another study by Flamant et al reported that EGFR transactivation mediates the tonic and fibrogenic effects of ET-1 in the mouse aorta (11). Transactivation of EGFR involves the cleavage of a latent form of heparin binding (Hb)-EGF by MMP or ADAM-like metalloproteinases (30, 33). Subsequent activation of
MAPK pathway then leads to increased collagen and fibronectin synthesis in mesangial cells (42). A recent report that investigated the growth response to ET-1 in glomerular mesangial cells using a microarray approach identified Hb-EGF as the main growth factor that was upregulated and EGF receptor antagonism of cells exposed to ET-1 inhibited the mesangial cell growth (25). This study also provided evidence that Hb-EGF levels are increased in the diabetic kidney. In light of these data, it is conceivable to speculate, but remains to be proven, that diabetes-induced activation of MMPs early in the disease process would promote cleavage of Hb-EGF which would then cause EGFR activation resulting in renal growth and fibrosis.

In summary, the GK model serves as a good model to study the slowly developing nephropathy observed in Type 2 diabetes. In contrast to decreased renal MMP activity in diabetic animal models that have significant renal impairment and mesangial matrix accumulation, early in the disease process MMPs are stimulated which in turn may lead to transactivation of EGF receptors by proteolytic processing of Hb-EGF. While the ET\textsubscript{A} receptor subtype is not involved in the early activation of renal MMPs in Type 2 diabetes, it contributes to transactivation of profibrotic EGFR.
ACKNOWLEDGMENTS

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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>
</tr>
<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>
</tr>
<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>
</tr>
<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>
</tr>
<tr>
<td>Body weight (mg)*</td>
<td>564 ± 41</td>
<td>607 ± 11</td>
<td>411 ± 39</td>
<td>409 ± 29</td>
</tr>
<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>
</tr>
<tr>
<td>Urine volume (ml/day)</td>
<td>17 ± 1</td>
<td>15 ± 2</td>
<td>16 ± 1</td>
<td>17 ± 1</td>
</tr>
<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>
</tr>
</tbody>
</table>

*p<0.05 GK vs control, **p<saline vs ABT-627*
Table 2. Total renal gelatinase activity (fluorescence/mg protein.min) in the control (C) and GK rats as determined by a fluorogenic substrate (n=5/group)

<table>
<thead>
<tr>
<th></th>
<th>Cortex</th>
<th>Medulla</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td>C</td>
<td>707 ± 126</td>
<td>213 ± 30</td>
</tr>
<tr>
<td>C + ABT-627</td>
<td>928 ± 102</td>
<td>163 ± 21</td>
</tr>
<tr>
<td>GK</td>
<td>1221 ± 102*</td>
<td>344 ± 43*</td>
</tr>
<tr>
<td>GK + ABT-627</td>
<td>1098 ± 171*</td>
<td>361 ± 25*</td>
</tr>
</tbody>
</table>

*p<0.05 vs C or C+ ABT-627
FIGURE LEGENDS

Fig. 1. Total 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 arrow. (B) Combined densitometric analysis of lytic bands corresponding to MMP-2 and pro-MMP2 in all the samples analyzed are shown by the bar graph and indicates an increase in total medullary MMP-2 activity that is not ameliorated by the ET$_A$ receptor blockade. Mean ± SEM of n=8/group, *p<0.05 vs control.

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 and indicated by the arrows. 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 ET$_A$ antagonism does not prevent this increase. Mean ± SEM of n=5/group, *p<0.05 vs control.

Fig. 3. Evidence for increased MT1-MMP and fibronectin in renal tissue. (A) Immunoreactive bands corresponding to 54 kDa MT1-MMP and (B) 40 kDa fibronectin were indicated by the arrows in a representative immunoblot. Densitometric analysis of immunoreactive bands indicates that MT1-MMP and fibronectin proteins are increased in diabetic tissue. Mean ± SEM of n=5/group, *p<0.05 vs control.
Fig. 4. Renal EGFR phosphorylation in increased in the GK rats. (A) Membranes were immunoblotted with native (EGFR) or phosphorylation-specific (EGFR~P) antibody and representative immunoblots are shown. Immunoreactive bands corresponding to 170 kDa EGFR was detected in most of the samples. The EGFR~P/EGFR ratio of the densitometric analysis of immunoreactive bands detected in all samples is shown in the bar graph and results indicate that the ratio is increased in both cortex and medulla of GK rats and ET$_A$ antagonism prevents EGFR transactivation. Mean ± SEM of n=5/group, *p<0.05 vs control, **p<0.05 vs GK.

Fig. 5. (A) Renal morphology in control and GK rats. The renal cross sections were stained with Masson trichrome and no pathological changes or significant collagen accumulation (blue staining) were observed either in the glomeruli (arrowhead) or tubulointerstitium (arrow). Representative of n=3 /group. (B) Real-time PCR analysis for collagen type I and IV gene expression in renal cortex. The amplicon intensity in GK and ABT-627-treated GK animals was normalized to that of control animals, which showed a 2 and 3-fold increase for collagen type I and IV, respectively, in diabetic animals and attenuation of this increase in treated rats. Mean ± SEM of n=3/group, *p<0.05 vs control, **p<0.05 vs GK.
Figure 1

A.

MMP-2

Cortex

C + ABT-627

GK

GK + ABT-627

B.

Optical density (pixels)

Cortex

Medulla

C + ABT-627

GK

GK + ABT-627

Optical density (pixels)

C

C + ABT-627

GK

GK + ABT-627

*
Figure 2
Figure 3

A. MT1-MMP

B. Fibronectin
Figure 4
A. - ABT-627 + ABT-627

Control

GK

B. Collagen I Collagen IV

Fold change in mRNA

Control GK GK + ABT-627

* **