Direct Evidence for Intrarenal Chymase-Dependent Angiotensin II Formation on the Diabetic Renal Microvasculature


Abstract—Our previous work supports a major role for angiotensin-converting enzyme (ACE)-independent intrarenal angiotensin (ANG) II formation on microvascular function in type 2 diabetes mellitus. We tested the hypothesis that there is a switch from renal vascular ACE-dependent to chymase-dependent ANGII formation in diabetes mellitus. The in vitro juxtaglomerular afferent arteriole (AA) contractile responses to the intrarenal conversion of the ACE-specific, chymase-resistant ANGII peptide ([Pro10]ANGII) to ANGII were significantly reduced in kidneys of diabetic (db/db) compared with control (db/m) mice. AA responses to the intrarenal conversion of the chymase-specific, ACE-resistant ANGI peptide ([Pro11, D-Ala12]ANGI) to ANGII were significantly enhanced in kidneys of diabetic compared with control mice. AA diameters were significantly reduced by 9±2, 15±3, and 24±3% of baseline in diabetic kidneys in response to 10, 100, and 1000 nmol/L [Pro11, D-Ala12]ANGI, respectively, and the responses were significantly attenuated by angiotensin type 1 receptor or chymase-specific (JNJ-18054478) inhibition. [Pro11, D-Ala12]ANGI did not produce a significant AA vasoconstriction in control kidneys. Chymase inhibition significantly attenuated ANGI-induced AA vasoconstriction in diabetic kidneys. Chymase inhibition significantly attenuated ANGII-induced AA vasoconstriction in diabetic, but not control kidneys. Renal vascular mast cell protease-4 or chymase/β-actin mRNA expression was significantly augmented by 5.1±1.4 fold; while ACE/β-actin mRNA expression was significantly attenuated by 0.42±0.08 fold in diabetic compared with control tissues. In summary, intrarenal formation of ANGII occurs primarily via ACE in the control, but via chymase in the diabetic vasculature. In conclusion, chymase-dependent mechanisms may contribute to the progression of diabetic kidney disease. (Hypertension. 2013;61:00-00.) ● Online Data Supplement

Key Words: afferent arteriole ▪ juxtaglomerular nephron ▪ db/db mouse ▪ angiotensin-converting enzyme ▪ chymase inhibitor ▪ JNJ-18054478

Classically, angiotensin-converting enzyme (ACE) is considered the major pathway for angiotensin (ANG) II formation. ACE-independent enzymatic pathways include serine proteases, tonin, cathepsin G, trypsin, and kallikrein. Evidence is mounting for an important role of chymase-dependent ANGII formation in human tissues: heart, vasculature, and kidney. Chymases are serine proteases that have chymotrypsin-like cleavage properties for the conversion of ANGII to ANGII at a rate 20 times greater than ACE. Human chymase has been identified as an efficient ACE, selectively hydrolyzing ANGII at Phe8 to generate bioactive ANGII. Mouse mast cell protease-4 is the functional homolog to human chymase, and ACE and mouse mast cell protease-4 cleave ANGII at identical sites to generate ANGII. Chymase inhibitors have emerged as potential therapeutic agents for treating various inflammatory, allergic, cardiovascular, and renal disorders. Therefore, ACE inhibitor monotherapy may allow for the continued generation of ANGII via ACE-independent pathways. The current studies were performed to investigate alternative intrarenal ANGII forming pathways that may be enhanced in the diabetic kidney with the overall goal of identifying new targets for treatment of diabetic kidney disease.

Recently, there has been growing interest in the role of chymase in various renal pathophysiologic states. Increased chymase expression has been observed in humans with diabetic nephropathy (DN), IgA nephropathy, autosomal dominant polycystic kidney disease, and hypertensive nephropathy, suggesting a central role of chymase in many forms of kidney disease in humans. Interestingly, in patients with DN, the number of renal chymase-positive mast cells is positively correlated with the severity of DN, suggesting that degranulation of mast cells promotes renal inflammation and fibrosis. Increased chymase expression in mesangial and vascular smooth muscle cells in human DN indicates...
that chymase is important for progression of the disease and suggests that pharmacological blockade of chymase may provide beneficial effects.

The current studies were performed in the db/db mouse (BKS.Cg-Dock7m +/+ Leprdb/J), which is an animal model of type 2 diabetes mellitus exhibiting features of human DN. Our previous study, as well as those of the research group of Batlle et al, have demonstrated a significant decrease in ACE protein expression and activity in the diabetic db/db compared with the control db/m kidney. Plasma and kidney ANGII levels were similar in db/db and db/m mice suggesting an augmentation of alternative ANGII forming enzymatic pathways in the db/db mouse. In spite of reduced ACE activity, afferent arterioles (AA) of control and diabetic kidneys responded with a similar magnitude of vasoconstriction to the intrarenal conversion of bath applied ANGI to ANGII. In kidneys of control mice, AA vasoconstrictor responses were mediated by ACE-dependent conversion of ANGI to ANGII; in contrast, AA vasconstrictor responses in diabetic mice were mediated by serine protease-dependent conversion of ANGI to ANGII. The rationale for conducting the current studies was to provide direct evidence for chymase as the specific serine protease responsible for ANGII formation in diabetic renal vascular disease.

We tested the hypothesis that there is a switch from renal ACE-dependent to chymase-dependent ANGII formation in diabetic vascular disease. Renal AA vascular responses to the intrarenal enzymatic conversion of ACE-specific and chymase-specific ANGI analogs to ANGII were determined in the absence or presence of a chymase inhibitor to determine the specific serine protease-dependent enzyme responsible for the intrarenal conversion of ANGI to ANGII in normal and diabetic kidneys. Quantification of vascular ACE and chymase mRNA expression was performed to provide support for the determination of the predominant intrarenal ANGII forming enzymes on vascular function in the type 2 diabetic kidney.

**Methods**

An extended Methods section is available in the online-only Data Supplement.

**Table.**  
<table>
<thead>
<tr>
<th>ANG Peptide Amino Acid Sequences</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANGI (1–8)</td>
<td>Asp-Arg-Val-Tyr-Ile-His-Pro-Phe</td>
</tr>
<tr>
<td>ANGI (1–10)</td>
<td>Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu</td>
</tr>
<tr>
<td>[Pro11, D-Ala12] ANGI (1–10)</td>
<td>Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Pro–</td>
</tr>
<tr>
<td>[Pro10] ANGI (1–10)</td>
<td>Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Pro–</td>
</tr>
</tbody>
</table>

ANG indicates angiotensin; ACE, angiotensin-converting enzyme.

Vascular responses to [Pro10] ANGI were used to determine functional ACE activity in control and diabetic mouse kidneys. [Pro10] ANGI is a 10 amino acid carboxyl-terminal ANGI analogue with substitution of proline for leucine at position 10. [Pro10] ANGI is an inactive precursor that yields ANGII when incubated with ACE, but not chymase.

Vascular responses to [Pro11, D-Ala12] ANGI were used to determine functional chymase activity in control and diabetic mouse kidneys. [Pro11, D-Ala12] ANGI is an ANGI analog that is an inactive precursor that yields ANGII when incubated with chymase, but not ACE. The 12 amino acid ANGI analog contains a penultimate proline, which prevents carboxy-terminal cleavage of the peptide by ACE, and carboxy-terminal D-alanine, which prevents carboxy-terminal degradation of the peptide by carboxy-peptidases.

**Animals**

Experiments were performed in adult male control db/m (n=38, Dock7m Leprb) and diabetic db/db (n=38, BKS.Cg-Dock7m +/+ Leprdb/J; #000642) mouse littermates.

### Mouse In Vitro Blood Perfused Juxtamedullary Nephron Technique

Experiments were conducted using the mouse in vitro blood perfused juxtamedullary nephron technique as we have previously reported in detail. AA diameters were measured during the following protocols:

1. **ACE-specific, chymase-resistant ANGI** ([Pro10] ANGI) peptide (Table). AA diameters were measured during superfusion with [Pro10] ANGI to determine the vascular effects of intrarenal conversion of ANGI to ANGII by ACE in kidneys of diabetic (n=7) and control (n=7) mice.

2. **Chymase-specific, ACE-resistant ANGI** ([Pro11, D-Ala12] ANGI) peptide (Table). AA diameters were measured during superfusion with [Pro11, D-Ala12] ANGI to determine the vascular effects of intrarenal conversion of ANGI to ANGII by chymase in kidneys of diabetic (n=8) and control (n=9) mice.

3. **Chymase-specific, ACE-resistant ANGI** ([Pro11, D-Ala12] ANGI) peptide in the presence of angiotensin type 1 (AT1) receptor blockade. Kidneys were superfused with an AT1 receptor antagonist followed by [Pro11, D-Ala12] ANGI in diabetic (n=7) and control (n=5) mice.

4. **Chymase-specific, ACE-resistant ANGI** ([Pro11, D-Ala12] ANGI) peptide in the presence of chymase inhibition. Kidneys were exposed to [Pro11, D-Ala12] ANGI in the presence of chymase-specific inhibitor in diabetic mice (n=3).

5. **ANGI in the presence of chymase inhibition.** Kidneys of diabetic (n=6) and control (n=6) mice were exposed to human ANGI in the continued presence of chymase inhibition.

### Quantitative Assessment of Renal Arterial Vascular Tissues

The renal arterial vasculature was isolated from diabetic and control mice as described by Schneider et al, and Western blot and real-time polymerase chain reaction were performed on the tissues.

### Data Analyses and Statistics

AA luminal diameters were measured manually and continuously using a digital image-shearing monitor. One-way repeated-measures ANOVA, 2-way ANOVA followed by Dunnett or Bonferroni test, paired t test, or unpaired t test were used as appropriate. P≤0.05 was considered statistically significant. Values are means±SEM.
Results

Baseline Parameters
Body weight was significantly higher in 18-week-old adult male diabetic (48.7±0.07 g; n=38) compared with control (32.2±0.04 g; n=38) mouse littermates. Baseline AA diameters of kidneys from diabetic mice (14.6±0.5 μm; n=31) were significantly larger than AAs from control (13.0±0.5 μm; n=27) mice.

AA Vasoconstrictor Responses to ACE-Specific [Pro10]ANGI
Figure 1 demonstrates the AA vasoconstriction to the ACE-specific, chymase-resistant ANGI peptide ([Pro10]ANGI; 0–1000 nmol/L). Figure 1A illustrates the average AA responses plotted in microns, and Figure 1B illustrates the average AA responses plotted as the delta % of baseline to the chymase-specific, ACE-resistant ANGI peptide ([Pro10]ANGI) in kidneys from control and diabetic mice. Significant AA vasoconstriction to 10, 100, 1000 nmol/L [Pro10]ANGI was observed in kidneys of control (−12±2, −19±4, −30±3%; n=7), but not diabetic (n=7) mice. [Pro10]ANGI produced a significantly greater response in AAs from control compared with diabetic mice.

AA Vasoconstrictor Responses to Chymase-Specific [Pro11, D-Ala12]ANGI
Figure 2A demonstrates the AA vasoconstriction plotted as the delta % of baseline to the chymase-specific, ACE-resistant ANGI peptide ([Pro11, D-Ala12]ANGI; 0–1000 nmol/L). Significant AA vasoconstriction to 10, 100, 1000 nmol/L [Pro11, D-Ala12]ANGI was observed in kidneys of diabetic (−9±2, −15±3, −24±3%; n=8), but not control (n=9) mice. [Pro11, D-Ala12]ANGI was observed in kidneys of control (−12±2, −19±4, −30±3%; n=6) and diabetic (n=7) mice. In the presence of chymase inhibition plotted in microns (Figure 3A), there was a significant difference between the AA diameter responses of diabetic and control kidneys. AA vasoconstrictor responses to [Pro11, D-Ala12]ANGI were significantly attenuated by AT1 receptor blockade in both groups (Figure 2B).

AA Responses to Chymase-Specific [Pro11, D-Ala12]ANGI in the Presence of AT1 Receptor Blockade
Pretreatment with an AT1 receptor blocker (100 μmol/L can-desartan) did not produce a change from the baseline diameter in either group. The AA vasoconstrictor responses to [Pro11, D-Ala12]ANGI were significantly attenuated by AT1 receptor blockade in both groups (Figure 2B).

AA Responses to Chymase-Specific [Pro11, D-Ala12]ANGI in the Presence of Chymase Blockade
AA diameters of diabetic kidneys did not change in response to [Pro11, D-Ala12]ANGI (1000 nmol/L: 4±2%, n=3) in the presence of chymase inhibition (data not shown). AA vasoconstrictor responses to [Pro11, D-Ala12]ANGI were significantly attenuated by chymase blockade compared with [Pro11, D-Ala12]ANGI alone in kidneys of diabetic mice (data not shown).

AA Responses to ANGI in the Presence of Chymase Blockade
Figure 3 illustrates the average AA responses to ANGI in the presence of chymase inhibition plotted in microns (Figure 3A) and delta % of baseline (Figure 3B) in kidneys from control and diabetic mice. In the presence of chymase inhibition, significant AA vasoconstriction to 10, 100, 1000 nmol/L ANGI (−12±2, −19±4, −30±3%; n=6) was observed in kidneys of control mice. However, chymase inhibition significantly attenuated the AA vasoconstriction to 10, 100, 1000 nmol/L ANGI (−7±2, −9±2, −11±4%; n=6) in kidneys of diabetic mice. In the presence of chymase inhibition, ANGI produced

Figure 1. Afferent arteriole (AA) diameter responses (A, μm; B, delta % of baseline) to the angiotensin-converting-enzyme (ACE)-specific, chymase-resistant (angiotensin I) ANGI peptide, ([Pro10]ANGI; 0–1000 nmol/L) in kidneys of control (∅, n=7) and diabetic (●, n=7) mice. AAs from control mice responded with a significant reduction in diameter with increasing concentrations of [Pro10]ANGI (P<0.05), whereas arterioles from diabetic mice did not respond. There was a significant difference between the AA diameter responses of diabetic and control kidneys. *P<0.05 vs baseline diameter; †P<0.05 control vs diabetic.

Figure 2. Afferent arteriole (AA) diameter responses (delta % of baseline) to the angiotensin-converting-enzyme (ACE)-resistant ANGI peptide ([Pro11, D-Ala12]ANGI; 0–1000 nmol/L) in the absence (A, solid lines) and presence of angiotensin type 1 (AT1) receptor blockade (ARB, B, dashed lines) in kidneys from control (∅, n=5) and diabetic (●, n=8, 7) mice. AAs from diabetic mice responded with a significant reduction in diameter with increasing concentrations of [Pro11, D-Ala12]ANGI (P<0.05), whereas arterioles from control mice did not respond. There was a significant difference between the AA diameter responses of diabetic and control kidneys. AA vasoconstriction produced by [Pro11, D-Ala12]ANGI in the diabetic kidney was significantly attenuated by AT1 receptor blockade. *P<0.05 vs baseline diameter; †P<0.05 control vs diabetic; #P<0.05 vs [Pro11, D-Ala12]ANGI alone.

Figure 3. Illustrates the average AA responses to ANGI in the presence of chymase inhibition plotted in microns (Figure 3A) and delta % of baseline (Figure 3B) in kidneys from control and diabetic mice. In the presence of chymase inhibition, significant AA vasoconstriction to 10, 100, 1000 nmol/L ANGI (−12±2, −19±4, −30±3%; n=6) was observed in kidneys of control mice. However, chymase inhibition significantly attenuated the AA vasoconstriction to 10, 100, 1000 nmol/L ANGI (−7±2, −9±2, −11±4%; n=6) in kidneys of diabetic mice. In the presence of chymase inhibition, ANGI produced...
a significantly greater response in AAs from control compared with diabetic mice.

At the conclusion of protocols 1 and 2, AA contractile responsiveness to ANGII remained intact in control (−12±2%; n=11) and diabetic (−11±1%; n=10) kidneys (Figure S1A in the online-only Data Supplement). In the continued presence of AT1 receptor blockade, norepinephrine produced a rapid and significant vasoconstriction in AAs of control and diabetic kidneys (−46±4, −35±6%; n=5, 7, respectively; Figure S1B). AA contractile responsiveness to ANGII remained intact in control (−24±2%; n=10) and diabetic (−18±2%; n=9) kidneys at the conclusion of protocols 2 and 5 (Figure S1C). The magnitude of the AA vasoconstrictions produced by ANGII, norepinephrine, and ANGII were not significantly different between kidneys of control and diabetic mice (Figure S1).

Quantitative Assessment of Renal Arterial Vascular Tissues

The renal vascular tissue isolation procedure yielded a significant 14.2±0.5-fold enrichment of α-smooth muscle actin (α-SMA) protein expression compared with renal cortical tissues from control mice (Figure 4A and 4B). Renal cortical tissue ACE protein expression was 16.5±0.7-fold enriched compared with renal vascular tissues from control mice (Figure 4C and 4D). Renal vascular tissues isolated from diabetic mice expressed significantly augmented mouse mast cell protease-4 (chymase) mRNA expression of 5.1±1.4-fold (Figure 4E) and attenuated ACE mRNA expression of 0.42±0.08 fold (Figure 4F) compared with control mice.

**Discussion**

Current drug therapies for the treatment of diabetic renal disease may slow the progression of the damage, but do not stop disease progression or restore normal kidney function for these patients. Despite the widespread use of inhibitors of the renin-angiotensin system and glucose-lowering medications, the incidence of diabetes-related end-stage renal disease continues to rise steadily indicating the need for the continued search for the mechanisms involved in the development and progression of DN.

Chymase has received considerable attention as an ACE-independent means to produce ANGII. It has been shown that the formation of ANGII from ANGI in coronary arteries is independent means to produce ANGII. It has been shown that the conversion of ANGI to ANGII is of similar magnitude in diabetic and control kidneys. AA vasoconstriction produced by the intrarenal conversion of ANGII was not attenuated by ACE inhibition, but was significantly attenuated by serine protease inhibition. Our earlier studies used ACE and nonspecific inhibitors to assess the role of chymase in diabetic renal vasculature.
serine protease inhibitors as a means to determine the major enzymatic pathways for intrarenal conversion of exogenously applied ANGI. Sequences for the synthesis of ANGI peptide analogs were obtained from the work of Husain et al.28,36 The ANGI analogs contain specific amino acid sequences that make them substrate-specific for either ACE or chymase enzymatic activity that allowed for the quantitative assessment of renal microvascular functional responses to the intrarenal conversion of these analogs to ANGII. ANGII synthesized via ACE and chymase-dependent pathways within the renal endothelium, microvasculature, glomerulus, tubules, and interstitium may act in an autocrine/paracrine manner via binding to plasma membrane AT1 receptors located on AA vascular smooth muscle cells.

The efficiency of human heart chymase for peptides with proline in the P2 position of ANGI is decreased by 95% compared with ANGII,27 indicating that this analog is chymase-resistant. Furthermore, the positive inotropic response of [Pro10]ANGI on hamster papillary muscle was completely suppressed by captopril pretreatment,36 indicating that the analog is specific for ACE. In our study, AA vasoconstriction to the ACE-specific, chymase-resistant [Pro10]ANGI was significantly greater in control than diabetic mice confirming significantly reduced ACE activity in the diabetic kidney. The significant AA responses to the ACE-specific, chymase-resistant ANGI analog confirm our previous work22 demonstrating that ACE is the predominant ANGI forming enzyme in the normal kidney.

It is well known that peptides with a proline in the penultimate position prevent ACE from cleaving a dipeptide from the carboxy terminus. The addition of a carboxy-terminal D-Ala prevents carboxypeptidases from making the penultimate proline into a carboxy-terminal proline. Hoit et al.38 cleverly combined these strategies to synthesize an ANGI analog that is resistant to ACE and carboxypeptidases, [Pro11, D-Ala12]ANGI, and allows for the in vitro and in vivo quantification of chymase activity. Li et al.29 demonstrated a lack of vasoconstrictor responses to [Pro11, D-Ala12]ANGI in mesenteric arteries of mast cell deficient Kitw/Kitw-v mice, whereas mesenteric arteries of control mice produced a vasoconstriction similar in magnitude to ANGII in the diabetic kidney. The maximal AA vasoconstriction to [Pro11, D-Ala12]ANGI in the diabetic kidney was of similar magnitude as we previously reported for ANGI,22 suggesting that for the duration of the experiment the analog is converted to ANGII as effectively as ANGI. Husain et al.38 demonstrated that the cardiovascular effects of [Pro11, D-Ala12]ANGI were not impacted by ACE inhibition in the conscious baboon and mouse.28 Additionally, the control kidney has minimal chymase activity. A significantly greater chymase and reduced ACE mRNA expression was detected in the renal vasculature of diabetic compared with control mice. The renal vascular isolation technique yielded an enhanced α-smooth muscle actin and diminished ACE protein expression compared with cortical tissues demonstrating a significant enrichment of vascular tissue and minimal tubular tissue in the protein and RNA extracts. These data are consistent with the microvascular physiological functional studies for intrarenal ANGII formation and support our hypothesis that there is a switch from ACE-dependent to chymase-dependent activity in the diabetic kidney.

Our previous work demonstrated a significant reduction in the density of renal cortical tubular ACE immunohistochemical staining and cortical ACE activity in diabetic compared with control mice,25,35 which is consistent with the work of Ye et al.23 In further studies, Ye et al.24 reported that the percentage of glomeruli with strong endothelial ACE staining was significantly greater in 8-week-old female db/db mice compared with control mice, which conflicts with our functional data in 18-week-old male db/db mice. Of interest are the findings of Soler et al.39 in which the percent of renal vessels demonstrating strong endothelial ACE immunostaining was increased in streptozotocin-induced type 1 diabetic compared with control mice. It is not clear whether the age, sex, or type of diabetic model influences renal arterial endothelial ACE protein expression.

The vasoconstrictor responses to conversion of [Pro11, D-Ala12] to ANGII in the diabetic kidney are attributed to AT1 receptor activation because the responses were blocked by ANG receptor blocker. In addition, the vasoconstrictor responses to conversion of [Pro11, D-Ala12] to ANGII are attributed to intrarenal chymase activity because these responses were blocked by the chymase inhibitor. These data implicate the importance of chymase as the primary route of formation of ANGII from ANGI in diabetic kidneys.

Most importantly, key data suggest that the AA vasoconstriction induced by intrarenal conversion of the endogenous form of ANGI to ANGII is significantly attenuated by a specific chymase inhibitor in kidneys of diabetic mice, but not in kidneys of control mice. The potent phosphinate chymase inhibitor, INJ-18054478, complexes with mammalian chymases and exhibits a potency of 0.07 μmol/L against human and macaque chymases and 5 μmol/L for guinea pig and hamster chymases.40 The 10 μmol/L dose of the chymase inhibitor, INJ-18054478, produced a complete inhibition of the AA vasoconstriction produced by intrarenal conversion of the chymase-specific, ACE-resistant [Pro11, D-Ala12]ANGI to ANGII in the diabetic kidney providing strong support for the efficacy of this inhibitor in the mouse kidney. This is the first study to document the efficacy of the chymase inhibitor, INJ-18054478, to block ANGII formation in the mouse renal vasculature.

At the conclusion of the [Pro10]ANGI and [Pro11, D-Ala12] ANGI protocols, bath application of ANGI or ANGII produced a significant vasoconstriction in AAs of control and diabetic kidneys indicating that the intrarenal ANGII forming enzymatic machinery and vascular smooth muscle cell AT1 receptor-mediated contractile properties were intact. In the presence of ANG receptor blockade, norepinephrine produced a potent vasoconstriction in control and diabetic kidneys indicating that although the AAs did not respond to bath applied ANGI analog, vascular smooth muscle cell vasoconstriction was not diminished. The maintenance of renal microvascular vasoconstrictor potential of AAs from both diabetic and
control kidneys provides support for the significant differences observed between AAs from diabetic and control kidneys in response to the enzyme-specific ANGI analogs.

The magnitude of the AA vasoconstriction of the diabetic kidney to 1 μmol/L [Pro1, D-Ala12]ANGI (~24±3%) was very similar to the magnitude of the vasoconstriction of the control kidney in response to 1 μmol/L ANGI in the presence of chymase inhibition (~30±3%). These data suggest that there is a similar magnitude of ANGII formation by chymase-dependent and ACE-dependent pathways in diabetic and control kidneys, respectively. Recent studies have shown that chymase inhibition protects against renal dysfunction in type 1 diabetic hamsters. In addition, chymase (mouse mast cell protease-4) deficient mice exhibit lower proteinuria, blood creatinine, and urea nitrogen levels, and less severe renal damage compared with wild-type mice indicating an aggravating role of renal chymase in glomerulonephritis disease progression.

The most significant finding of the present study is the identification of chymase as the major ACE-independent pathway for the formation of ANGII in the type 2 diabetic leptin-receptor deficient mouse kidney. In the diabetic kidney, AA vasoconstriction to intrarenally formed ANGII from the substrate ANGI is blocked by inhibition of chymase activity. In contrast, intrarenally formed ANGII from the substrate ANGI produces a potent AA vasoconstriction in the presence of chymase inhibition in the control kidney. Our studies may provide a potential mechanism involved in the superior renoprotective effects of combining an ACE inhibitor with an AT1 receptor antagonist relative to ACE inhibitor therapy alone in patients with DN, which has been reported in some clinical studies. In addition, the presence of this ACE-independent pathway for ANGII formation may explain the continued proteinuria in some patients on maximal ACE inhibitor therapy. However, the ONTARGET trial indicated that in patients with cardiovascular disease or diabetes mellitus, the combination of ANG receptor blocker and ACE inhibitor provided more adverse events without an increase in benefit compared with either monotherapy. We suggest that ACE inhibitor monotherapy may allow for the continued generation of ANGII via chymase-dependent pathways, which contributes to fibrosis, proteinuria, and reduced renal function in diabetic patients.

Perspectives

Despite the first-line use of ACE inhibitors and ANG receptor blockers for the treatment of DN, there is still a large need to improve therapies for the prevention of DN and dramatically reduce the rates of disease progression for these patients. Our studies support a major role for chymase-dependent ANGII formation in the db/db renal vasculature and thus provide a novel translational approach to human disease. Chymase inhibition may provide substantial renal protection in diabetic patients. Physicians may treat with an ANG receptor blocker when diabetic renal disease patients are unresponsive to ACE inhibition. Treatment with an ANG receptor blocker may provide additional benefit because of the inhibition of ANGII produced by ACE-dependent and chymase-dependent pathways. Targeting chymase as a therapeutic target for chronic kidney disease patients with normal blood pressure may provide the advantage of reducing intrarenal chymase-dependent fibrosis, proteinuria, and vasocostriction without causing systemic hypotension that can lead to further reductions in glomerular filtration rate and renal blood flow, which is often observed with treatment with ACE inhibitors or ANG receptor blockers.

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Disclosures

None.

References

Renal Chymase-Dependent ANGII in Diabetes

What Is New?

- Our compelling data demonstrate a major role for chymase-dependent ANGII formation on renal microvascular function in the diabetic kidney.

What Is Relevant?

- Because currently available therapies do not arrest disease progression, it is imperative that new therapeutic approaches be investigated for the prevention and treatment of kidney disease in diabetic patients.

Novelty and Significance

These are the first studies to indicate a significant contribution of chymase to the intrarenal formation of ANGII on afferent arteriolar function in the diabetic kidney. Future studies will test the ability of chymase inhibitors, specifically N60-18054478, to attenuate the in vivo disease-related changes in diabetic renal function.
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DIRECT EVIDENCE FOR INTRARENAL CHYMASE-DEPENDENT ANGIOTENSIN II FORMATION ON THE DIABETIC RENAL MICROVASCULATURE

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ONLINE-ONLY DATA SUPPLEMENT

METHODS

Animals. The procedures used in this study were approved by the Animal Care and Use Committee of Louisiana State University Health Sciences and conducted according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Experiments were performed in adult male (18-wk-old) control db/m (n=38, Dock7m Leprdb) and diabetic db/db (n=38, BKS.Cg-Dock7m +/+ Leprdb/J; #000642) mouse littermates (The Jackson Laboratory). Adult male Sprague-Dawley rats (452 ± 13 g BW, n=58; Charles River Laboratories) were used as blood donors for the study of the mouse renal microvasculature. All animals were provided ad libitum access to food and water during the study.

Mouse in vitro blood perfused juxtamedullary nephron technique. Experiments were conducted using the mouse in vitro blood perfused juxtamedullary nephron technique as we have previously reported in detail.1-3 Kidneys were studied under euglycemic (5 mmol/L) and hyperglycemic (30 mmol/L glucose) incubation conditions (5% BSA perfusion solution, 1% BSA superfusion solution, rat plasma) for control and diabetic mice, respectively.2,3 Donor blood was collected from anesthetized rats. Peptides and drugs were added to the 5% BSA perfusion solution and/or 1% BSA superfusion and/or rat plasma. A minimum of 15 min was allowed for equilibration of the renal vasculature upon initiation of the blood perfusion. Baseline AA diameters were measured during control conditions (1% BSA solution superfusion, 5 min). ANGI peptides (1, 10, 100, and 1000 nmol/L) were applied to kidney via the 1% BSA superfusion solution for a period of 5 min for each dose. Each protocol was followed by a 15 min recovery period.

AA diameters were measured during the following protocols:

1) ACE-specific, chymase-resistant ANGI ([Pro10]ANGI) peptide (Table). AA diameters were measured during superfusion with [Pro10]ANGI to determine the vascular effects of intrarenal conversion of ANGI to ANGII by ACE in kidneys of diabetic (n=7) and control (n=7) mice. The response to 1 µmol/L human ANGI was determined in the same vessels at the conclusion of the experiment.

2) Chymase-specific, ACE-resistant ANGI ([Pro11, DAla12]ANGI) peptide (Table). AA diameters were measured during superfusion with [Pro11, DAla12]ANGI to determine the vascular effects of intrarenal conversion of ANGI to ANGII by chymase in kidneys of diabetic (n=8) and control (n=9) mice. The response to 1 µmol/L ANGI or 100 nmol/L human ANGII was determined in the same vessels at the conclusion of the experiment.

3) Chymase-specific, ACE-resistant ANGI ([Pro11, DAla12]ANGI) peptide in the presence of AT1 receptor blockade. Kidneys were superfused with an AT1 receptor antagonist (100 µmol/L candesartan) for 10 min followed by [Pro11, DAla12]ANGI in diabetic (n=7) and control (n=5) mice. Since it was expected that the AT1 receptor antagonist would block the vasoconstriction resulting from intrarenally formed ANGII, the vasoconstrictor response to 1 µmol/L norepinephrine (NE, 3 min) was examined in the same vessels at the conclusion of the experiment.
4) Chymase-specific, ACE-resistant ANGI ([Pro₁¹, DAla₁²]ANGI) peptide in the presence of chymase inhibition. Diabetic mice received an i.p. injection of the chymase-specific inhibitor JNJ-18054478⁶ (50 mg/kg) at 30 min prior to kidney harvesting. Chymase-specific inhibitor (JNJ-18054478, 10 µmol/L final concentration) was also added to the perfusion and superfusion solutions to ensure continuous chymase blockade throughout the entire experiment. Kidneys were exposed to [Pro₁¹, DAla₁²]ANGI in the presence of chymase-specific inhibitor in diabetic mice (n=3) since kidneys of control mice do not respond to this ANGI analog.

5) ANGI in the presence of chymase inhibition. Kidneys of diabetic (n=6) and control (n=6) mice were exposed to human ANGI in the continued presence of chymase inhibition (see protocol 4). The response to 100 nmol/L ANGII was determined in the same vessels at the conclusion of the experiment.

Reagents. [Pro¹⁰]ANGI (Bachem Americas, Inc.), [Pro₁¹,DAla₁²]ANGI (Biopeptide Co., Inc.), and JNJ-18054478 (Janssen Pharmaceutical) were prepared from lyophilized reagents and dissolved in 0.9% saline on the day of the experiment. Stock solutions of ANGI (# 002-01, Phoenix Pharmaceuticals), ANGII (#002-12, Phoenix Pharmaceuticals), and candesartan (AstraZeneca) were dissolved in 0.9% saline, stored at -20°C, and diluted on the day of the experiment. NE (Abbott Laboratories) was diluted in Tyrode solution on the day of the experiment.

Quantitative assessment of renal arterial vascular tissues. The renal arterial vasculature was isolated from diabetic and control mice as described by Schneider et al.⁷ Mice were anesthetized, kidneys removed and decapsulated, placed in ice-cold sterile normal saline, and pressed through a circle sieve (100 µm pore). The renal tissue was viewed repeatedly under a high-power stereomicroscope until the vascular tree was devoid of tubular structures. The vascular tissues were stored at -80°C until protein or RNA extraction. Western blot analysis was performed on 10 µg or 20 µg of renal vascular (n=4) and cortical (n=4) tissue extracts from control mice as we have previously described⁸ using primary antibodies for α-smooth muscle actin (α-SMA, anti-α-actin mouse mAb (1A4), 1:200, Calbiochem # 113200), mouse ACE specific goat IgG (1:1,000 AF1513; R&D system), and β-actin (β-actin antibody (C4), 1:1,000, Santa Cruz # sc-47778 HRP). Densitometry was performed using Image J analysis software (NIH). Quantitative real-time RT-PCR (qRT-PCR) was performed on 50 ng of total RNA in triplicate to evaluate mMCP-4 and ACE mRNA as previously described ⁹. Data were normalized based on the expression level of the β-actin mRNA. The sequences were as follows—mMCP-4: forward primer, 5′-GAAGTGAAA AGCCTGACCTGC -3′; reverse primer, 5′-ATTCTGTCTTGCTCACATCA -3′; probe, 5′-AATCACTGTCACCCTTGG AGCTC-3′; ACE: forward primer, 5′-AGGGACATGTGGGCGCAGAC-3′; reverse primer, 5′-CGGTGGGCTTCTCTAATCGA-3′; and β-actin: forward primer, 5′-TGTGATGGTGGAATGGGTGCAGAA-3′; reverse primer, 5′-TGTGATGGGCCAGATCTTCTCCATGT-3′.

Data analyses and statistics. AA luminal diameters were measured manually and continuously throughout the protocol at a single site along the length of the AA using a digital image-shearing monitor ¹⁻³. The average diameter (µm) during the control (5 min), ANGI or ANGI analogs (5
min), AT₁ receptor antagonist (final 5 min), NE (3 min), and recovery (final 5 min) periods was used for one-way repeated-measures or two-way ANOVA followed by Dunnett's or Bonferroni's test (Sigma Stat 3.5, Systat Software, Inc.). Because of the significant difference in baseline AA diameters between control and diabetic mice, two-way ANOVA was conducted on the percent change from the baseline diameter for the AA responses to all peptides and drugs. Paired or unpaired t-test was used as appropriate. p ≤ 0.05 was considered statistically significant. Values are means ± SEM.

REFERENCES


Figure S1. Significant AA diameter responses (delta % of baseline) to 1 µmol/L ANGI (A; n=11 11) following recovery from the responses to [Pro10]ANGI or [Pro11,D-Ala12]ANGI, or 1 µmol/L norepinephrine (NE; B, n=5, 7) in the presence of AT1 receptor blockade, or 0.1 µmol/L ANGII (C, n=10, 9) following recovery from the responses to [Pro11,D-Ala12]ANGI or ANGI in the presence of the chymase-specific inhibitor, JNJ-18054478 in kidneys from control (□) and diabetic (■) mice. There were no significant differences in the magnitude of the AA vasoconstrictor responses to ANGI, NE, or ANGII in control compared to diabetic mice. * p≤0.05 vs. recovery diameter