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 ANGII peptide ([Pro11, D-Ala12]ANGII) 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]ANGII, respectively, and the responses were significantly attenuated by angiotensin type 1 receptor or chymase-specific (JNJ-18054478) inhibition. [Pro11, D-Ala12]ANGII did not produce a significant AA vasoconstriction in control kidneys. Chymase inhibition significantly attenuated ANGII-induced AA vasoconstriction in diabetic, but not control kidneys. Renal vascular mouse 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;61:465-471.) ● Online Data Supplement

Key Words: afferent arteriole ■ juxtamedullary nephron ■ db/db mouse ■ angiotensin-converting enzyme ■ chymase ■ 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.1 Evidence is mounting for an important role of chymase-dependent ANGII formation in human tissues2,3: heart,4 vasculature,5 and kidney.6,7 Chymases are serine proteases that have chymotrypsin-like cleavage properties for the conversion of ANG to ANGII at a rate 20 times greater than ACE.8,9 Human chymase has been identified as an efficient ACE, selectively hydrolyzing ANGII at Phe8 to generate bioactive ANGII.8 Mouse mast cell protease-4 or chymase/β-actin mRNA expression was 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]ANGII, respectively, and the responses were significantly attenuated by angiotensin type 1 receptor or chymase-specific (JNJ-18054478) inhibition. [Pro11, D-Ala12]ANGII did not produce a significant AA vasoconstriction in control kidneys. Chymase inhibition significantly attenuated ANGII-induced AA vasoconstriction in diabetic, but not control kidneys. Renal vascular mouse 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;61:465-471.) ● Online Data Supplement

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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.22,23–25 Our previous study,22 as well as those of the research group of Batlle et al.,23–25 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 mice.22 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.22 In kidneys of control mice, AA vasoconstrictor responses were mediated by ACE-dependent conversion of ANGI to ANGII; in contrast, AA vasoconstrictor responses in diabetic mice were mediated by serine protease-dependent conversion of ANGI to ANGII.22 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.

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.22,26–27 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=5).
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 mouse kidneys as described by Schneider et al.20 and Western blot21 and real-time-polymerase chain reaction22 were performed on the tissues.

Data Analyses and Statistics

AA luminal diameters were measured manually and continuously using a digital image-shearing monitor.22,26–27 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 mean±SEM.

<table>
<thead>
<tr>
<th>Table. ANG Peptide Amino Acid Sequences</th>
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<tr>
<td><strong>ANG Peptides</strong></td>
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<td>ANGI (1–8)</td>
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<td>ANGI (1–10)</td>
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<td>[Pro10] ANGI (1–10)</td>
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<td>[Pro11, D-Ala12] ANGI (1–12)</td>
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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 carboxy-terminal ANGI analogue with substitution of proline for leucine at position 10.36 [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.36
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 AA diameters 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 ([Pro11, D-Ala12]ANGI; 0–1000 nmol/L). Significant AA vasoconstriction to 10, 100, 1000 nmol/L [Pro10]ANGI was observed in kidneys of control (−12±2, −18±5, −16±4%; n=7), but not diabetic (n=7) mice. [Pro10]ANGI produced a significantly greater response in 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 [Pro11, D-Ala12]ANGI in kidneys from control and diabetic mice. Significant AA vasoconstriction to 10, 100, 1000 nmol/L [Pro11, D-Ala12]ANGI alone in kidneys of control (−12±2, −19±4, −30±3%; n=6) was observed in kidneys of diabetic (n=8), but not control (n=9) mice. [Pro11, D-Ala12]ANGI produced a significantly greater response in AAs from diabetic compared with control mice.

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 candesartan) 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±3, −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 AT_1 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 formation of ANGII from ANGI in coronary arteries is dependent on both ACE and nonspecific (cross-hatched, n=4) and cortex (gray bars, n=7) α-smooth muscle actin (α-SMA; A, B) and angiotensin-converting-enzyme (ACE; C, D) protein expression in tissues harvested from control mice. The vascular tissue isolation technique results in an enrichment of vascular and a diminution of tubular tissues. Renal vascular mouse mast cell protease (mMCP)-4 (E) and ACE (F) mRNA expression in tissues harvested from control (◼, n=7) and diabetic (●, n=7) mice. Data are factored for the β-actin protein or mRNA expression and expressed relative to cortical or vascular tissues or control mice. There is a switch from ACE to chymase mRNA expression in the diabetic renal vasculature. *P≤0.05 vascular vs cortical, #P≤0.05 cortical vs vascular; †P≤0.05 control vs diabetic.

Figure 4. Representative immunoblots (A, C) and quantitative analysis (B, D) of renal vessels (gray bars, n=4) and cortex (cross-hatched, n=4) α-smooth muscle actin (α-SMA; A, B) and angiotensin-converting-enzyme (ACE; C, D) protein expression in tissues harvested from control mice. The vascular tissue isolation technique results in an enrichment of vascular and a diminution of tubular tissues. Renal vascular mouse mast cell protease (mMCP)-4 (E) and ACE (F) mRNA expression in tissues harvested from control (◼, n=7) and diabetic (●, n=7) mice. Data are factored for the β-actin protein or mRNA expression and expressed relative to cortical or vascular tissues or control mice. There is a switch from ACE to chymase mRNA expression in the diabetic renal vasculature. *P≤0.05 vascular vs cortical, #P≤0.05 cortical vs vascular; †P≤0.05 control vs diabetic.
serine protease inhibitors as a means to determine the major enzymatic pathways for intrarenal conversion of exogenously applied ANG I. Sequences for the synthesis of ANG I peptide analogs were obtained from the work of Husain et al. The ANG I 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 ANG II. ANG II synthesized via ACE and chymase-dependent pathways within the renal endothelium, microvascularity, glomerulus, tubules, and interstitium may act in an autocrine/paracrine manner via binding to plasma membrane AT
associated with minimal tubular tissue in the protein and RNA extracts. These data are consistent with the microvascular physiological functional studies for intrarenal ANG II 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, which is consistent with the work of Ye et al. In further studies, Ye et al 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 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 [Pro
, D-Ala
] to ANG II in the diabetic kidney are attributed to AT
receptor activation because the responses were blocked by ANG receptor blocker. In addition, the vasoconstrictor responses to conversion of [Pro
, D-Ala
] to ANG II are attributed to intrarenal ANG II vasoconstriction because these responses were blocked by the chymase inhibitor. These data implicate the importance of chymase as the primary route of formation of ANG II from ANG I in diabetic kidneys.

Most importantly, key data suggest that the AA vasoconstriction induced by intrarenal conversion of the endogenous form of ANG II 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 5 μmol/L for guinea pig and hamster chymases. 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 [Pro
, D-Ala
] ANG II to ANG II 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 ANG II formation in the mouse renal vasculature.

At the conclusion of the [Pro
]ANG I and [Pro
, D-Ala
] ANG I protocols, bath application of ANG I or ANG II produced a significant vasoconstriction in AAs of control and diabetic kidneys indicating that the intrarenal ANG I forming enzymatic machinery and vascular smooth muscle cell AT
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 ANG I 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 ANGII analogs.

The magnitude of the AA vasoconstriction of the diabetic kidney to 1 μmol/L [Pro14, D-Ala15]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**

Novelty and Significance

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.

Summary

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 JNJ-18054478, to attenuate the renal microcirculation at a renoprotective combination.


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

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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 Lepr<sup>db</sup>) and diabetic db/db (n=38, BKS.Cg-Dock7<sup>m</sup> +/- Lepr<sup>db</sup>/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 ([Pro<sup>10</sup>]ANGI) peptide (Table). AA diameters were measured during superfusion with [Pro<sup>10</sup>]ANGI<sup>4</sup> 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 ([Pro<sup>11</sup>, DAla<sup>12</sup>]ANGI) peptide (Table). AA diameters were measured during superfusion with [Pro<sup>11</sup>, DAla<sup>12</sup>]ANGI<sup>5</sup> 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 ([Pro<sup>11</sup>, DAla<sup>12</sup>]ANGI) peptide in the presence of AT<sub>1</sub> receptor blockade. Kidneys were superfused with an AT<sub>1</sub> receptor antagonist (100 µmol/L candesartan) for 10 min followed by [Pro<sup>11</sup>, DAla<sup>12</sup>]ANGI in diabetic (n=7) and control (n=5) mice. Since it was expected that the AT<sub>1</sub> 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 ([Pro\textsuperscript{11}, DAla\textsuperscript{12}]ANGI) peptide in the presence of chymase inhibition.** Diabetic mice received an i.p. injection of the chymase-specific inhibitor JNJ-18054478\textsuperscript{6} (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 [Pro\textsuperscript{11}, DAla\textsuperscript{12}]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.** [Pro\textsuperscript{10}]ANGI (Bachem Americas, Inc.), [Pro\textsuperscript{11},DAla\textsuperscript{12}]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.\textsuperscript{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 described\textsuperscript{8} 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 .\textsuperscript{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'-AGGGAA CATGTGGGCGCAGAC-3'; reverse primer, 5'-CGGTGGGCTTCTCAACATCGA-3'; and β-actin: forward primer, 5'-TGTGATGGTGGAATGGGTGTCAGAA-3'; reverse primer, 5'-TGTGGTGCCAGATCTTCCATGT-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 \textsuperscript{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. \( p \leq 0.05 \) 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) following recovery from the responses to [Pro\(^{10}\)]ANGI or [Pro\(^{11},\text{d-Ala}^{12}\)]ANGI, or 1 µmol/L norepinephrine (NE; B, n=5, 7) in the presence of AT_1 receptor blockade, or 0.1 µmol/L ANGII (C, n=10, 9) following recovery from the responses to [Pro\(^{11},\text{d-Ala}^{12}\)]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.