Enhanced Vascular Chymase-Dependent Conversion of Endothelin in the Diabetic Kidney

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ABSTRACT
Background: Diabetic nephropathy (DN) is associated with enhanced renal, plasma, and urinary endothelin (ET)-1 levels. Chymase cleaves Big ET-1 (1-38) to ET-1 (1-31), which is further cleaved by neutral endopeptidase to ET-1 (1-21). The current study tested the hypothesis that afferent arterioles (AA) of diabetic kidneys exhibit enhanced vasoconstrictor responses to chymase-dependent intrarenal ET formation compared to control kidneys.

Methods: In situ juxtamedullary AA vasoconstrictor responses to the intrarenal conversion of Big ET-1 (1-38) to ET-1 (1-21) were performed in the absence and presence of chymase inhibition in type 2 diabetic db/db and control db/m mice studied under in vitro experimental conditions.

Results: AA vasoconstrictor responses to Big ET-1 (1-38) were significantly enhanced in diabetic compared to control kidneys. In the presence of chymase inhibition (JNJ-18054478), AA vasoconstrictor responses of diabetic kidneys to Big ET-1 (1-38) were significantly less than the responses of control kidneys. AA diameters decreased similarly to ET-1 (1-21) in diabetic and control kidneys.

Conclusions: AA responses to the intrarenal conversion of Big ET-1 (1-38) to ET-1 in the absence of chymase enzymatic activity were significantly reduced in kidneys of diabetic compared to control mice, while the magnitude of the vasoconstriction to ET-1 (1-21) was not different. These data suggest that AA vasoconstriction produced by the chymase-dependent pathway is significantly greater in diabetic compared to control kidneys. We propose that intrarenal chymase-dependent ET-1 production contributes to the decline in function and progression to end-stage renal disease in patients with type 2 diabetes.

INTRODUCTION
Our overall goal is to identify new therapeutic targets for the prevention, treatment, and reversal of diabetic renal disease. Our recent studies support a role for increased chymase activity in the renal vasculature of type 2 diabetic db/db mice and thus provide a novel translational approach to human disease. Chymase inhibition may provide substantial kidney protection in diabetic patients and offer an effective approach to decrease pain and suffering, improve the quality of human life, and significantly reduce morbidity and mortality in this growing patient population.

Diabetes and Obesity
In the United States (US), 26 million people are diabetic and 57 million people are prediabetic, placing them at higher risk for the development of diabetes. The number of cases of diabetic kidney disease increased 34% from 1988 to 2008 in the US in spite of the increasing use of glucose-lowering medications and renin-angiotensin system (RAS) inhibitors. Diabetes is the sixth leading cause of death in the US. Type 2 diabetes mellitus, the most common endocrine disease, affects 8% of the US population, and obesity has been identified as the principal risk factor associated with the rising prevalence of type 2 diabetes. The incidence of obesity and diabetes has reached epidemic proportions, justifying the importance of identifying novel pathways and molecular targets for the prevention and reversal of this horrible disease. Because 80% of cases of type 2 diabetes are related to obesity, we selected an obese model of type 2 diabetes, the db/db mouse.

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Keywords: Chymases, diabetic nephropathies, endothelin-1, kidney failure, vasoconstriction

Funding: This work was supported by the American Heart Association Grant-In-Aid 2250875 (Harrison-Bernard).
Diabetic Nephropathy and Current Therapies

Diabetes is the number one cause of end-stage renal disease; therefore, protection against end-organ damage is imperative. Pharmacologic drugs that inhibit the actions of angiotensin-converting enzyme (ACE) and the angiotensin type 1 (AT1) receptor are registered for delaying the onset and slowing the progression of diabetic nephropathy (DN) in humans; however, these drugs do not halt disease progression to end-stage kidney failure. These treatments do not consistently reduce proteinuria, which is not only a powerful predictor, but also a promoter of renal progression. These findings indicate the need for additional therapeutic targets that may provide further benefit when used alone or in combination with current therapies.

Endothelin System

The endothelin (ET) system plays a well-recognized, powerful role in blood pressure regulation and sodium and fluid homeostasis. The ET system participates in the normal regulation of the renal microcirculation and in the handling of sodium and water. It also contributes to the pathogenesis of renal injury. The ET system is activated in hypertension, diabetic kidney disease, pulmonary hypertension, systemic sclerosis, and cancer. ET-1 is an endothelial cell-derived peptide that is one of the most potent mammalian vasoconstrictor peptides known to date. ET-1 production normally occurs in the endothelium; however, vascular smooth muscle cell synthesis of ET-1 is thought to occur in cardiovascular disease. ET-1 messenger RNA (mRNA) encodes a 212-amino-acid prepropeptide that is cleaved to yield the proET-1 peptide. ProET-1 is cleaved to yield Big ET-1 (1-38). The conversion of Big ET-1 (1-38) to ET-1 (1-21) occurs primarily through the action of endothelin-converting enzyme (ECE); however, chymase can cleave Big ET-1 (1-38) to ET-1 (1-31), which is further cleaved by neutral endopeptidase (NEP) to the biologically potent ET-1 (1-21) (Figure 1). Every cell type in the kidney expresses ET receptors. ETA receptors predominate on vascular smooth muscle, while endothelin B receptors are found on endothelial cells. The kidney is exquisitely sensitive to ET-1, having up to 10-fold greater sensitivity to the vascular effects of the peptide as compared to other organ beds. The chymase and NEP pathways may constitute a therapeutically valid target toward the ET system in vascular diseases.

ET and Diabetes

Humans with type 1 and type 2 diabetes mellitus exhibit elevated plasma ET-1 levels and increased urinary ET-1 excretion, demonstrating an activated ET system. Kidney ET-1 gene transcription is increased in type 1 diabetes in the rat. Evidence for an additional antiproteinuric effect of ET A receptor blockade was recently reported for DN patients given RAS blockade. The significantly elevated urinary ET-1 excretion in diabetic db/db compared to control mice indicates that this mouse model replicates the human disease.

Chymase

Mast cells are present in virtually every vascularized tissue of the human body, including the kidney. Shi and colleagues have provided key evidence for a critical role of mast cells in the progression of diet-induced obesity and diabetes in mice. The increased chymase expression observed in humans with DN, IgA nephropathy, autosomal dominant polycystic kidney disease, and hypertensive nephropathy suggests a central role of chymase in many forms of kidney disease in humans. Increased
mast cells could promote unwanted fibrosis and inflammation that ultimately lead to destruction of kidney structure.

Classically, ACE is considered the major pathway for angiotensin II (AngII) formation; however evidence is mounting for an important role of chymase-dependent AngII formation in human tissues.\textsuperscript{34-36} Chymases are serine proteases that have chymotrypsin-like cleavage properties for conversion of angiotensin I to AngII at a rate 20 times greater rate than that of ACE.\textsuperscript{27,28} Therefore, increased chymase-dependent AngII formation can occur under conditions of normal or reduced ACE activity. Chymase also contributes to the formation of ET-1 (1-21) from Big ET-1 (1-38). Chymases influence structural remodeling through their ability to activate transforming growth factor beta (TGF-\beta) and interleukin-1, degrade extracellular matrix proteins, and stimulate collagen fibrillogenesis. Human chymase activity is inhibited by serine protease inhibitors and chymostatin but is not affected by ACE inhibitors.\textsuperscript{28} In the mouse, mMCP-4 \(\beta\)-chymase promotes AngII generation.\textsuperscript{29} Hypertension develops in mice with overexpression of rat vascular chymase in smooth muscle cells, indicating that chymase generates vasoconstrictive products that are thought to be AngII and ET-1.\textsuperscript{30} Recent studies show that chymase inhibition protects against renal dysfunction in type 1 diabetic hamsters.\textsuperscript{31} Chymase (mMCP-4)-deficient mice exhibit lower proteinuria, blood creatinine, and blood urea nitrogen levels and less severe renal damage in a model of glomerulonephritis, indicating an aggravating role of renal chymase in disease progression.\textsuperscript{32} Interestingly, the number of renal chymase-positive mast cells is positively correlated with the development of DN in humans.\textsuperscript{33} These studies support a role for chymase in renal disease progression, but the exact mechanism of the protection from loss of the enzymatic properties of chymase is unknown.

### ET System/Chymase

Mice with combined knockout of ECE-1 and ECE-2 have appreciable ET-1 levels,\textsuperscript{34} suggesting that other proteases are involved in ET-1 formation. The fact that Big ET-1 can be converted to ET-1 (1-31) by chymase raises the interesting possibility that alternative processing of Big ET-1 is of biological and pathophysiological relevance (Figure 1). An improved understanding of the mechanisms involved in the intrarenal synthesis of ET-1 in DN may lead to novel therapeutic targets of the kidney ET system by inhibitors of chymase used alone or in combination with RAS or ET receptor blockers.

In the current study, we extended our observations of the chymase-dependent effects on AngII formation\textsuperscript{1} to determine the chymase-dependent activity on conversion of Big ET-1 (1-38) to ET-1 (1-31) and ultimately to ET-1 (1-21)-induced vasoconstriction in diabetic and control kidneys with the goal of determining the potential role of chymase in the pathogenesis of DN, one of the most dreaded consequences of diabetes. We tested the hypothesis that the diabetic kidney exhibits enhanced chymase-dependent conversion of Big ET-1 (1-38) to the potent vasoconstrictor, profibrotic, and proteinuric peptide ET-1.

### METHODS

#### Animals

The procedures used in this study were approved by the Animal Care and Use Committee of Louisiana State University Health Sciences Center and conducted according to the Public Health Service Policy on the Humane Care and Use of Laboratory Animals. Experiments were performed in adult male 18-week-old control db/m (n=11, Dock7\textsuperscript{m} Lepr\textsuperscript{db}) and diabetic db/db (n=14, BKS.Cg-Dock7\textsuperscript{m} +/+ Lepr\textsuperscript{db}/J; #000642) mouse littermates (Jackson Laboratory, Bar Harbor, ME). Adult male Sprague-Dawley rats (n=25; Charles River Laboratories, Raleigh, NC) were used as blood donors for the study of the mouse renal microvasculature. All animals had ad libitum access to food and water during the study.

#### Mouse In Vitro Blood Perfused Juxtamedullary Nephron Technique

We conducted experiments using the mouse in vitro blood perfused juxtamedullary nephron technique as previously reported in detail.\textsuperscript{35-37} Kidneys were studied under euglycemic (5 mmol/L glucose) and hyperglycemic (30 mmol/L glucose) incubation conditions (5% bovine solution albumin [BSA] perfusion solution [Calbiochem, #12659], 1% BSA superfusion solution, rat plasma) for control and diabetic mice, respectively.\textsuperscript{36,37} Donor blood was collected from anesthetized rats. Chymase inhibitor JNJ-18054478 was added to the 1% and 5% BSA perfusion solutions and 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). ET peptides were applied to the kidney via the 1% BSA superfusion solution for a period of 5 min for each dose. Each protocol was followed by a 10-min recovery period. The protocols were as follows:

**AA Responses to ET-1 (1-21):** AA diameters were measured during superfusion with 1 pmol/L to 10 nmol/L ET-1 (1-21) to determine the vasoconstrictor effects of vascular smooth muscle cell \(\text{ET}_A\) receptor...
stimulation in the kidneys of diabetic (n=2) and control (n=2) mice.

AA Responses to Big ET-1 (1-38). AA diameters were measured during superfusion with 10 pmol/L to 100 nmol/L Big ET-1 (1-38) to determine the vascular effects of intrarenal conversion of Big ET-1 (1-38) to ET-1 (1-31) and ET-1 (1-21) in the kidneys of diabetic (n=8) and control (n=6) mice. The AA response to 100 nmol/L ET-1 (1-21) was determined in the same vessels at the conclusion of the experiment.

AA Responses to Big ET-1 (1-38) in the Presence of Chymase Inhibition. Diabetic and control mice received an intraperitoneal injection of the chymase-specific inhibitor JNJ-18054478 (50 mg/kg) 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 ET-1 (1-31) and ET-1 (1-21) in the kidneys of diabetic (n=8) and control (n=6) mice. The AA response to 100 nmol/L ET-1 (1-21) was determined in the same vessels at the conclusion of the experiment.

AA Responses to Big ET-1 (1-38) in the Presence of Chymase Inhibition. Figure 3A demonstrates the AA vasoconstriction plotted as the delta % of baseline to Big ET-1 (1-38) in kidneys from control and diabetic mice. In the presence of chymase inhibition, significant AA vasoconstriction of 50%–3% was observed in response to 100 nmol/L Big ET-1 (1-38) in control kidneys, while AAs decreased by 31%–5% in kidneys of diabetic mice. In the absence of chymase activity, Big ET-1 (1-38) produced a significantly greater vasoconstrictor response in AAs from diabetic compared to control mice. The AA vasoconstrictor response to 100 nM ET-1 (1-21) (Figure 3B) was similar in diabetic (–47% ± 5%) and control (–40% ± 5%) kidneys.

RESULTS
Baseline Parameters

Body weight was significantly higher in 18-week-old adult male diabetic (49.2 ± 2.2 g; n=14) compared to control (32.5 ± 0.6 g; n=11) mouse littermates. Baseline AA diameters of kidneys from diabetic mice (16.4 ± 0.9 μm) were significantly larger than arterioles from control (14.2 ± 0.8 μm) mice (Figure 2A).

AA Responses to ET-1 (1-21). The AA responses to ET-1 (1-21) were comparable in diabetic and control kidneys so the data were combined. Figure 2B demonstrates a significant AA vasoconstriction to 0.01 to 10 nmol/L ET-1 (1-21; n=4). AA diameter decreased by 23%–2% and 44%–2% in response to 1 and 10 nM ET-1 (1-21), respectively.

AA Responses to Big ET-1 (1-38). Figure 3A demonstrates the AA vasoconstriction plotted as the delta % of baseline to Big ET-1 (1-38) in kidneys of control and diabetic mice. Significant AA vasoconstriction of 20%–5% was observed in response to 100 nmol/L Big ET-1 (1-38) in control kidneys, while AAs decreased by 29%–5% in kidneys of diabetic mice. Big ET-1 (1-38) produced a significantly greater vasoconstrictor response in AAs from diabetic compared to control mice. The AA vasoconstrictor response to 100 nM ET-1 (1-21) (Figure 3B) was similar in diabetic (–40% ± 6%) and control (–39% ± 5%) kidneys.

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. The average diameter (μm) during 5-min periods was used for 1-way repeated-measures or 2-way analysis of variance (ANOVA) followed by Holm-Sidak test (Sigma Stat 3.5, Systat Software, Inc., Chicago, IL). Because of the significant difference in baseline AA diameters between control and diabetic mice, 2-way ANOVA was conducted on the percentage change from the baseline diameter for the AA responses to all peptides and drugs. Paired or unpaired t test was used as appropriate, and P<0.05 was considered statistically significant. Values are presented as mean ± standard error of the mean (SEM).

Figure 2. Baseline juxtamedullary AA diameter (A, μm) in kidneys of control (■, n=11) and diabetic (□, n=14) mice. Mouse AAs responded with a significant reduction in diameter (B, delta % of baseline) with increasing concentrations of ET-1 (1-21). #P<0.05 vs control; *P<0.05 vs baseline diameter. AA, afferent arterioles; ET, endothelin.
DISCUSSION

We hypothesized that the diabetic kidney exhibits enhanced chymase-dependent conversion of Big ET-1 (1-38) to the potent vasoconstrictor, profibrotic, and proteinuric peptide ET-1. The ultimate goal of these basic science experiments is to propose that targeting of chymase may provide blockade of diabetic-disease-associated, chymase-dependent formation of ET-1.

Our recent studies demonstrate increased AA vasoconstriction due to enhanced intrarenal chymase-dependent AngII formation. The enhanced chymase-dependent effects occur in the diabetic renal vasculature that expresses a 5-fold increase in chymase mRNA expression and 50% reduction in ACE mRNA expression compared to control kidneys. The present study demonstrates that intrarenal conversion of Big ET-1 (1-38) to ET-1 produced a significantly greater AA vasoconstrictor response in kidneys of diabetic compared to control mice. These results suggest that the diabetic renal vasculature is capable of a greater formation of ET-1 due to enhanced enzymatic activity of ET-forming proteins.

There is no difference in the ET-1 (1-21)-mediated AA vasoconstrictor response in diabetic compared to control responses, suggesting that the AA expression of ET receptors is not different between the two groups of mice. These studies support an enhanced enzymatic formation of ET in the diabetic kidney by chymase. It is possible that there are alterations in the enzymatic formation of ET-1 (1-21) by ECE and NEP in the diabetic kidney; however, we did not determine the contribution of ECE and NEP activity to the enhanced renal vascular formation of ET in these studies.

AA vasoconstrictor responses to Big ET-1 (1-38) were significantly reduced in the presence of chymase inhibitor in diabetic compared to control kidneys. The reduced AA vasoconstrictor responses were not dependent on a different functional expression of ET because the AA responses to ET-1 (1-21) were similar in the two groups. The large AA vasoconstrictor responses to ET-1 (1-21) document maintained vascular responsiveness to ET-1 (1-21) in the presence of chymase inhibition of intrarenal ET peptide synthesis. A strength of these in vitro studies is that they allow us to test the physiological responsiveness of the diabetic and control renal microvasculature in response to the intrarenal synthesis of ET-1 (1-38) and ET-1 (1-31) through the enzymatic activity of chymase.

Our data in control mice are in agreement with Schneider et al, who demonstrated a significant AA

**Figure 3.** Significant AA diameter responses (delta % of baseline) to Big ET-1 (1-38) (A) and ET-1 (1-21) (B) in kidneys from control (■, n=6) and diabetic (●, n=8) mice. AAs from control and diabetic mice responded with a significant reduction in diameter with increasing concentrations of Big ET-1 (1-38). There was a significant difference between the AA diameter responses of diabetic and control kidneys to Big ET-1 (1-38), but not to ET-1 (1-21). *P<0.05 vs baseline diameter; †P<0.05 control vs diabetic. AA, afferent arterioles; ET, endothelin.

**Figure 4.** Significant AA diameter responses (delta % of baseline) in the presence of chymase inhibition to Big ET-1 (1-38) (A) and ET-1 (1-21) (B) in kidneys of control (■, n=3) and diabetic (●, n=4) mice. AAs from diabetic mice responded with a significantly smaller reduction in diameter with increasing concentrations of Big ET-1 (1-38) in the presence of the chymase-specific antagonist JNJ-18054478 compared to control mice. There was a significant difference between the AA diameter responses to Big ET-1 (1-38), but not to ET-1 (1-21) in the presence of chymase inhibition between diabetic and control kidneys. *P<0.05 vs baseline diameter; †P<0.05 control vs diabetic. AA, afferent arterioles; ET, endothelin.
vessels to Big ET-1 (1-38) in rat juxtaglomerular nephrons indicative of intrarenal formation of ET-1 in rat kidneys. Big ET-1 (1-38) had less vasoconstrictor activity than equimolar concentrations of ET-1 (1-21) in rat AAs as we observed in the mouse AAs. Watts et al. have reported chymase-dependent processing of Big ET-1 (1-38) in the rat aorta; chymase-positive immunostaining did not colocalize with mast cells. Recent studies by Simard et al. demonstrate that chymase is significantly involved in the pressor response caused by conversion of intravenously administered Big ET-1 (1-38) to ET-1 (1-21) in the mouse in vivo. Identification of a key role for chymase was concluded with the use of a specific peptidic chymase inhibitor. Renal ET-1 mRNA expression is elevated in Zucker Diabetic Fatty rats compared to control rats. Taken together, in vivo and in vitro studies support a role for chymase-dependent ET-1 formation in the vasculature.

The ASCEND study was a large, international trial that assessed the effects of an ET receptor antagonist added to continued ACE and/or angiotensin receptor blocker treatment on DN that was terminated early because of excessive rates of adverse events. We propose that chymase may be a key enzyme in the formation of ET-1 in diabetic kidney disease. A subpopulation of DN patients who are resistant to the beneficial effects of ACE inhibition may respond positively to chymase inhibition.

CONCLUSIONS
AA responses to the intrarenal conversion of Big ET-1 (1-38) to ET-1 in the absence of chymase enzymatic activity were significantly reduced in kidneys of diabetic compared to control mice, while the magnitude of the vasoconstriction to ET-1 (1-21) was not different. These data suggest that AA vasoconstriction produced by the chymase-dependent pathway is significantly greater in diabetic compared to control kidneys. We propose that intrarenal chymase-dependent ET-1 production contributes to the decline in function and progression to end-stage renal disease in patients with type 2 diabetes.

ACKNOWLEDGMENT
The authors are grateful for the excellent and informative scientific discussions with Drs David Pollock and Pedro d’Orleans-Juste regarding the experimental design for these studies.

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This article meets the Accreditation Council for Graduate Medical Education and the American Board of Medical Specialties Maintenance of Certification competencies for Patient Care and Medical Knowledge.