Inhibition of chymase protects against diabetes-induced oxidative stress and renal dysfunction in hamsters

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1Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, 2Innovation Center for Medical Redox Navigation, Kyushu University, Fukuoka; 3Department of Internal Medicine, Fukuoka University, Chikushino Hospital, Chikushino; and 4Department of Pharmacology, Faculty of Medicine, Kagawa University, Kagawa, Japan

Submitted 8 June 2010; accepted in final form 23 September 2010.

Maeda Y, Inoguchi T, Takei R, Sawada F, Sasaki S, Fujii M, Kobayashi K, Urata H, Nishiyama A, Takayanagi R. Inhibition of chymase protects against diabetes-induced oxidative stress and renal dysfunction in hamsters. Am J Physiol Renal Physiol 299: F1328–F1338, 2010. First published September 29, 2010; doi:10.1152/ajprenal.00337.2010.—Accumulating evidence suggests that the intrarenal renin-angiotensin system may be involved in the progression of diabetic nephropathy. Chymase is a potent local angiotensin II-forming enzyme in several species, including humans and hamsters. However, the pathophysiological role of chymase is not fully understood. Here, we report a causal role of chymase in diabetic nephropathy and the therapeutic effectiveness of chymase inhibition. In the present study, renal chymase expression was markedly upregulated in streptozotocin-induced diabetic hamsters. Oral administration of a specific chymase inhibitor, TEI-F00806, completely ameliorated proteinuria, the overexpression of transforming growth factor-β and fibronectin in glomeruli, and renal mesangial expansion, by normalizing the increase in intrarenal angiotensin II levels in diabetic hamsters independently of blood pressure levels. In contrast, ramipril did not show such sufficient effects. These effects occurred in parallel with improvements in superoxide production and expression of NAD(P)H oxidase components [NAD(P)H oxidase 4 and p22phox] in glomeruli. This study showed for the first time that chymase inhibition may protect against elevated intrarenal angiotensin II levels, oxidative stress, and renal dysfunction in diabetes. These findings suggest that chymase offers a new therapeutic target for diabetic nephropathy.

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Their kidneys were immediately excised and stored at ~80°C for the following experiments. For the next experiment, STZ-induced diabetic hamsters were orally administered with ramipril (5 mg·kg⁻¹·day⁻¹; in water) or chymase-specific inhibitors, TEI-E00548 or TEI-F00806 (10 mg·kg⁻¹·day⁻¹, respectively), for 8 wk. The dose of ramipril was selected to produce maximal ACE inhibition as determined by measurement of serum ANG II. The chymase inhibitors were kindly provided by Teijin Pharma (Tokyo, Japan). Heart rate and systolic blood pressure were measured in the left lower limb by a noninvasive modified tail-cuff method (BP Monitor for Rats and mice, model MK-2000, Muromachi Kikai, Tokyo, Japan). Urine collection was performed using a metabolic cage for 2 days at weeks 4 and 8. Urine samples were analyzed using ELISA kits for 8-hydroxy-2′-deoxyguanosine (8-OHdG; 8-OHdG Check, Japan Institute for the Control of Aging, Fukuroi, Japan) and a commercial kit for all protein levels (WAKO Pure Chemical Industries, Osaka, Japan), adjusted for 24-h urine volume and body weight. Hamsters used to evaluate RAS components were killed by decapitation to avoid changes in the circulatory system that may immediately affect the RAS, and the kidneys were removed. Serum samples were obtained at the same time for the following assays. (See below for methods used for glomerular isolation and DHE fluorescence.) The creatinine levels in serum and urine were also measured with a commercially available kit (Cayman Chemical, Ann Arbor, MI). This study was approved by the Animal Care and Use Committee, Kyushu University.

Immunohistochemistry. For light microscopic examination, kidneys were fixed in 10% formaldehyde and embedded in paraffin. Antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) with 0.1% Nonidet P-40 (Sigma-Aldrich) in a microwave oven. Triton X-100 (0.1%) was used for cell permeabilization. Endogenous peroxidase was inactivated with 3% H₂O₂ in methanol for 15 min at room temperature, and sections were preincubated for 30 min with 1% bovine serum albumin in PBS. Samples were incubated with rabbit polyclonal anti-hamster chymase antibody (8 μg/ml; Teijin Pharma), goat polyclonal anti-mouse ACE antibody (6 μg/ml; R&D Systems, Minneapolis, MN), anti-human NAD(P)H oxidase 4 (NOX4), p22phox, and fibronectin goat polyclonal antibodies (4 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), overnight at 4°C. Sections were incubated with goat anti-rabbit IgG antibody (1:5,000; GE Healthcare UK, Buckingham, UK) or sheep anti-mouse IgG antibody (1:5,000; GE Healthcare UK, Buckingham, UK) as a secondary antibody. We used the ECL Plus system (GE Healthcare UK) for detection. Detection of superoxide using dihydroethidium in situ. Dihydroethidium (DHE) staining was performed, as previously described with minor modifications (31). For the next experiment, STZ-induced diabetic hamsters were intravenously administered 1 ml of DHE (1 mg/ml in PBS; Invitrogen) through the right jugular vein under isoflurane anesthesia. Two hours after the DHE injection, the hamsters were killed by transcardiac perfusion with 50 ml of 4% formaldehyde in PBS. Their kidneys were frozen immediately in O.C.T. compound (Tissue-Tech II, Sakura Finechemical, Tokyo, Japan) and sectioned 10 μm thick on a cryostat. The kidney sections were followed by nuclear staining with Hoechst 33258 (Invitrogen) in PBS for 15 min in a dark chamber and mounted after the rinse in distilled H₂O. Fluorescence images were obtained using a fluorescence microscope (model BZ-9000, Keyence, Osaka, Japan). The relative fluorescence intensity in the glomeruli was quantified using Adobe Photoshop software (version 6.0, Adobe Systems, Mountain View, CA). We selected every glomerulus manually in all ×200 images and counted a mean value of histogram for a red-colored channel as a fluorescent level of DHE in each glomerulus. Then, the average of glomerular fluorescent levels in each image was compared between the groups.

Morphological and cytochemical examinations. Periodic acid-Schiff (PAS) staining (PAS Staining Kit, Muto Pure Chemicals, Tokyo, Japan) was performed for the morphological analysis of renal
injury. To objectively quantify the mesangial matrix accumulation, we measured the total glomeruli area and the PAS-positive area in cross sections through their vascular poles, according to a previously described technique (43). Briefly, images of glomeruli were manually clipped out from PAS-stained kidney sections and binarized at a fixed threshold level which distinguishes a PAS staining area using Adobe Photoshop software. Then, the PAS staining area was measured and adjusted by glomerular area in every glomerulus using ImageJ, an image-analysis software provided by the US National Institutes of Health. Means of these indexes of 10 glomeruli from each section were compared statistically.

Measurement of serum RAS components and kidney ANG II levels. We measured the serum RAS components using commercially available kits for ANG II (Angiotensin II Enzyme Immunoassay Kit, SPI-BIO, Montigny-le-Bretonneux, France), renin activity (Sensolyte 390 Renin Assay Kit, AnaSpec, Fremont, CA), ANG I (Angiotensin I EIA Kit, Phoenix Pharmaceuticals, Burlingame, CA), and ACE activity (Angiotensin-I Converting Enzyme Activity Assay kit, Life Laboratory, Yamagata, Japan) in accordance with the manufacturers’ instructions. Kidney ANG II was measured as previously described (28) using a combined method of solid-phase extraction and radioimmunoassay (RIA). Kidney samples were extracted on C18 reverse-phase bond eluting columns (1210–2032, Varian). RIA was performed using [125I]-ANG II (NEX-105, NEN Life Sciences) and ANG II antiseraum (RAB-002-12, Phoenix Pharmaceuticals) with commercially available standards.

Statistical analysis. All data are expressed as means ± SE. Statistical analysis was performed with Student’s t-test or one-way ANOVA with Fisher’s protected least significant difference test. P < 0.05 was considered statistically significant.

RESULTS

Chymase expression levels in diabetic kidney. We first investigated whether the expression of chymase is increased in renal tissues from STZ-induced diabetic hamsters. To elucidate the effect of hyperglycemia on chymase expression, we used diabetic hamsters with or without normoglycemia, which was achieved by intraperitoneal insulin infusion via osmotic minipumps. The characteristics of the experimental hamsters are shown in Table 1. After 4 wk of insulin infusion, the blood glucose levels were reduced to near-normal levels, while the untreated diabetic group showed severe hyperglycemia and body weight loss. Immunostaining analysis revealed that chymase expression was strongly enhanced in renal tubular cells and in glomeruli from untreated diabetic hamsters. Insulin infusion normalized the diabetes-induced increased expression of chymase (Fig. 1A). Real-time PCR analysis showed that mRNA levels for chymase were increased by 2.4-fold in the renal cortex and by 5.1-fold in the renal medulla from diabetic hamsters compared with controls. These increases were normalized by insulin infusion (Fig. 1B). In contrast, ACE expression was enhanced in renal tissues from diabetic hamsters, but was localized in the brush border of the proximal tubules (Fig. 1A), as shown in a previous study (20).

Effect of a chymase inhibitor on RAS components, blood pressure, and oxidative stress. Next, we used orally active chymase-specific inhibitors to evaluate whether the increased expression of chymase is related to renal dysfunction in diabetic hamsters. The detailed biochemical properties of TEI-E00548, which we used here, are reported elsewhere (18). Briefly, TEI-E00548 inhibits hamster chymase in vitro (Ki = 30.6 nM) and has little effect on other serine proteases including cathepsin G, elastase, chymotrypsin, and trypsin (concentration at 50% inhibition was >1 M). It did not inhibit ACE-dependent ANG II formation. We also used another specific inhibitor, TEI-F00806, whose inhibitory kinetics (Ki = 9.85 nM) is approximately three times stronger than that of TEI-E00548. Both inhibitors (10 mg·kg⁻¹·day⁻¹) were administered orally to the diabetic hamsters for 8 wk. The characteristics of the experimental hamsters are shown in Table 2. Administration of both chymase inhibitors did not affect the serum ANG II levels, but completely suppressed intrarenal ANG II overproduction in diabetic hamsters (Fig. 2A). In contrast, the administration of ramipril completely suppressed the serum ANG II levels, but did not affect intrarenal ANG II overproduction in diabetic hamsters. Consequently, ramipril significantly lowered blood pressure, whereas chymase inhibitors did not (Fig. 2, B and C). Interestingly, the chymase mRNA levels were only enhanced in diabetic hamsters and appeared to be suppressed by chymase inhibitors, while ACE mRNA levels were not altered (Fig. 2D).

To evaluate renal oxidative stress, we measured the expression of the standard oxidative stress marker 8-OHdG using and ELISAs to measure 24-h urinary excretion levels. Urinary 8-OHdG excretion levels were significantly higher in STZ hamsters (0.47 ± 0.07 µg·day⁻¹·100 g body wt⁻¹, P < 0.001 vs. control) than in control hamsters (0.07 ± 0.01) and was significantly reduced by TEI-F00806 (0.13 ± 0.02, P < 0.001 vs. STZ) and TEI-E00548 (0.24 ± 0.04, P < 0.001 vs. STZ), but not by ramipril (0.31 ± 0.10, P = 0.061 vs. STZ) (Fig. 3A). We also performed DHE staining for the detection of superoxide produced by NAD(P)H oxidase in the kidneys. In whole kidney section, STZ hamsters showed extremely high fluorescence levels of DHE in contrast to controls. This was distinctly ameliorated by chymase inhibitors, especially by TEI-F00806 (Fig. 3B, left). Interestingly, the localization of DHE fluorescence was clearly associated with the distribution of ACE and chymase in the kidney (Fig. 3B, middle and right). Then, DHE fluorescence intensity in glomeruli was quantified by densitometry analysis. Relative fluorescence intensity in glomeruli to controls was significantly higher in STZ hamsters (3.6-fold, P < 0.001), and this increase was significantly reduced by TEI-F00806 (1.0-fold to controls, P < 0.001 vs. STZ) and TEI-E00548 (1.3-fold to controls, P < 0.001 vs. STZ). Ramipril also decreased DHE fluorescence level (1.4-fold to controls, P < 0.001 vs. STZ), but it was yielded to TEI-F00806 (P = 0.023; Fig. 3C). These results revealed a massive increase

### Table 1. Characteristics of study animals

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>STZ</th>
<th>STZ + CIPPII</th>
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<tbody>
<tr>
<td>10 wk old (Before treatment)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Body weight, g</td>
<td>122.3 ± 8.7</td>
<td>123.3 ± 29.7</td>
<td>114.8 ± 29.6</td>
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<tr>
<td>Blood glucose, mg/dl</td>
<td>95.0 ± 28.8</td>
<td>218.0 ± 215.3*</td>
<td>274.2 ± 228.7*</td>
</tr>
<tr>
<td>14 wk old (After treatment)</td>
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<tr>
<td>Body weight, g</td>
<td>162.3 ± 17.5</td>
<td>129.0 ± 44.2‡</td>
<td>158.8 ± 31.4‡</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>94.7 ± 48.3</td>
<td>352.8 ± 227.7‡</td>
<td>81.9 ± 63.9‡</td>
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</table>

Values are means ± SD; n = 8 for all groups. STZ, streptozotocin (STZ)-induced diabetic hamsters with sham operation; STZ + CIPPII, STZ-induced diabetic hamsters with continuous intraperitoneal insulin injection (CIPPII). *P < 0.01 vs. control (ANOVA). ‡P < 0.01 vs. control and STZ + CIPPII (ANOVA). †P < 0.01 vs. before treatment (paired t-test).
in superoxide production in the diabetic kidneys and its attenuation by chymase inhibitors. In addition, we measured the expression of NAD(P)H oxidase, a major source of ROS production in the kidney. In parallel with the accumulation of oxidative stress markers, immunostaining analysis of NOX4, a major component of NAD(P)H oxidase, and the other component p22phox showed that the expression of NAD(P)H oxidase was remarkably increased in the glomeruli and renal tubules of diabetic hamsters.

Table 2. Characteristics of animals

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>STZ</th>
<th>STZ+Ramiplus</th>
<th>STZ+TEI-E00548</th>
<th>STZ+TEI-F00806</th>
</tr>
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<tbody>
<tr>
<td>10 wk old (Before treatment)</td>
<td></td>
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<td></td>
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<tr>
<td>Body weight, g</td>
<td>136.2 ± 5.3</td>
<td>116.6 ± 4.7†</td>
<td>123.6 ± 5.7</td>
<td>119.3 ± 4.5‡</td>
<td>126.8 ± 4.5</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>95.6 ± 6.8</td>
<td>287.2 ± 18.6†</td>
<td>266.3 ± 23.9†</td>
<td>276.8 ± 10.5†</td>
<td>286.2 ± 32.1†</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>369.9 ± 15.2</td>
<td>370.0 ± 19.3</td>
<td>417.6 ± 28.6</td>
<td>382.9 ± 31.6</td>
<td>411.9 ± 33.0</td>
</tr>
<tr>
<td>18 wk old (After treatment)</td>
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<tr>
<td>Body weight, g</td>
<td>178.5 ± 2.3*</td>
<td>165.3 ± 1.3*</td>
<td>130.7 ± 25.6†</td>
<td>146.0 ± 9.0</td>
<td>152.0 ± 16.0</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>109.8 ± 7.2</td>
<td>315.1 ± 19.8†</td>
<td>347.3 ± 42.3†</td>
<td>379.2 ± 18.2†</td>
<td>316.0 ± 21.9†</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>427.1 ± 35.4</td>
<td>369.9 ± 31.9</td>
<td>407.1 ± 43.1</td>
<td>391.0 ± 30.1</td>
<td>348.1 ± 38.2</td>
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<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.8 ± 0.1</td>
<td>1.3 ± 0.5</td>
<td>1.5 ± 0.3</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
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<tr>
<td>Serum creatinine clearance, ml/min</td>
<td>0.2 ± 0.0</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.0</td>
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<tr>
<td>Serum renin activity, µM/h</td>
<td>0.9 ± 0.2</td>
<td>1.8 ± 0.2†</td>
<td>1.9 ± 0.4†</td>
<td>1.6 ± 0.3</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>Serum ANG I, ng/ml</td>
<td>3.1 ± 0.1</td>
<td>4.7 ± 0.2†§</td>
<td>3.2 ± 0.5</td>
<td>3.9 ± 0.3</td>
<td>4.7 ± 0.5†§</td>
</tr>
<tr>
<td>Serum ACE activity, nmol°1·min°1</td>
<td>163.8 ± 16.9</td>
<td>271.9 ± 35.7†§</td>
<td>121.2 ± 14.5</td>
<td>261.2 ± 27.1†§</td>
<td>309.5 ± 16.7†§</td>
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</table>

Values are means ± SE; n = 8 for all groups. ACE, angiotensin-converting enzyme. *P < 0.01 vs. before treatment (paired t-test). †P < 0.01, ‡P < 0.05 vs. control (ANOVA). §P < 0.01 vs. STZ+Ramiplus (ANOVA with Fisher’s protected least significant difference test).
diabetic hamsters, and that these increases were reduced by chymase inhibitors (Fig. 4A). As shown in Fig. 4B, Western blot analyses confirmed that the protein levels of NOX4 and p22phox in the glomerular homogenates of diabetic hamsters were higher than those of controls (2.3-fold, \( P < 0.05 \); 2.7-fold, \( P < 0.01 \) (ANOVA). Non-significant \( P \) values are not indicated. *\( P < 0.05 \), **\( P < 0.01 \) (ANOVA). C: systolic blood pressure during the treatment period. \( \delta P < 0.01 \), \( \delta P < 0.05 \) vs. STZ with ramipril (ANOVA). Non-significant \( P \) values are not indicated. D: quantification of hamster chymase and ACE mRNA in glomerular homogenates by real-time PCR. White bars, nondiabetic controls (control); black bars, STZ-induced diabetic hamsters (STZ). Results are shown as a percentage of controls. Values are means ± SE. Non-significant \( P \) values are not indicated. *\( P < 0.05 \), **\( P < 0.01 \) (ANOVA).

Chymase inhibitors prevent diabetic renal injury. We next evaluated the effect of chymase inhibitors on the development of renal injury in diabetic hamsters. Untreated diabetic hamsters showed a 5.4-fold increase in urinary protein excretion levels (0.29 ± 0.05 \( \mu \)g·day\(^{-1}\)·100 g body wt\(^{-1}\), \( P < 0.001 \) vs. controls [0.05 ± 0.01]) after 4 wk of treatment, and a 7.2-fold increase at week 8 (1.12 ± 0.30, \( P < 0.001 \) vs. controls [0.16 ± 0.05]). Oral administration of TEI-E00548 partially reduced proteinuria in diabetic hamsters at week 8 of treatment (0.59 ± 0.21, \( P = 0.039 \) vs. STZ). Administration of TEI-F00806 completely reduced proteinuria at week 4 (0.07 ± 0.02, \( P < 0.001 \) vs. STZ) and by ~80% of the increase at week 8 (0.37 ± 0.12, \( P = 0.005 \) vs. STZ), whereas ramipril did not significantly improve proteinuria throughout the treatment period (Fig. 5A). We then investigated the effect of chymase inhibition on renal mesangial expansion, which is one of the most striking histological characteristics of diabetic nephropathy, after 8 wk of treatment. The glomerular structure in the diabetic hamsters exhibited accelerated mesangial expansion, which was characterized by an increased area of the PAS-positive mesangial matrix compared with that observed in control hamsters (Fig. 5B). PAS staining was evaluated as the mesangial matrix index (MMI), which was expressed as the ratio of mesangial area to the total glomerular area, and was computed by quantitative image-analysis methods. The MMI was markedly increased by 1.5-fold in the glomeruli of untreated diabetic hamsters (\( P < 0.001 \)) compared with that observed in control hamsters (Fig. 5B). PAS staining was evaluated as the mesangial matrix index (MMI), which was expressed as the ratio of mesangial area to the total glomerular area, and was computed by quantitative image-analysis methods. The MMI was markedly increased by 1.5-fold in the glomeruli of untreated diabetic hamsters (\( P < 0.001 \)) compared with that observed in control hamsters (Fig. 5B). PAS staining was evaluated as the mesangial matrix index (MMI), which was expressed as the ratio of mesangial area to the total glomerular area, and was computed by quantitative image-analysis methods. The MMI was markedly increased by 1.5-fold in the glomeruli of untreated diabetic hamsters (\( P < 0.001 \)) compared with that observed in control hamsters (Fig. 5B). PAS staining was evaluated as the mesangial matrix index (MMI), which was expressed as the ratio of mesangial area to the total glomerular area, and was computed by quantitative image-analysis methods. The MMI was markedly increased by 1.5-fold in the glomeruli of untreated diabetic hamsters (\( P < 0.001 \)) compared with that observed in control hamsters (Fig. 5B). PAS staining was evaluated as the mesangial matrix index (MMI), which was expressed as the ratio of mesangial area to the total glomerular area, and was computed by quantitative image-analysis methods. The MMI was markedly increased by 1.5-fold in the glomeruli of untreated diabetic hamsters (\( P < 0.001 \)) compared with that observed in control hamsters (Fig. 5B). PAS staining was evaluated as the mesangial matrix index (MMI), which was expressed as the ratio of mesangial area to the total glomerular area, and was computed by quantitative image-analysis methods. The MMI was markedly increased by 1.5-fold in the glomeruli of untreated diabetic hamsters (\( P < 0.001 \)) compared with that observed in control hamsters (Fig. 5B). PAS staining was evaluated as the mesangial matrix index (MMI), which was expressed as the ratio of mesangial area to the total glomerular area, and was computed by quantitative image-analysis methods. The MMI was markedly increased by 1.5-fold in the glomeruli of untreated diabetic hamsters (\( P < 0.001 \)) compared with that observed in control hamsters (Fig. 5B).
Effect of chymase inhibitors on ANG II-derived matrix protein synthesis. To elucidate the effects of chymase inhibition on the signaling pathway leading to renal mesangial expansion, we evaluated the expression of TGF-β1, a key cytokine that mediates extracellular matrix accumulation and glomerular expansion in diabetes (48), and fibronectin, a predominant matrix protein. Real-time PCR showed that the expression of TGF-β1 in glomerular homogenates from diabetic hamsters was 2.4-fold higher than that of controls ($P < 0.05$) and that this increase was significantly ($P < 0.05$; Fig. 6A) suppressed by TEI-F00806 only. Western blot analysis confirmed that the protein expression of TGF-β1 in the glomerular homogenates was 2.4-fold higher in the diabetic hamsters than in the controls ($P = 0.018$) and these increases were significantly reduced by both TEI-E00548 and TEI-F00806 to the control levels ($P = 0.010$ and 0.007 vs. STZ, respectively), but not by ramipril ($P = 0.914$ vs. STZ; Fig. 6, B and C). Immunostaining revealed that the expression of fibronectin was enhanced in glomeruli from diabetic hamsters and that this increase was reduced by TEI-E00548 and TEI-F00806, but not by ramipril (Fig. 6D).

DISCUSSION

In this study, we used the hamster diabetic models to evaluate the effect of chymase-specific inhibitors on diabetic nephropathy. Human and hamster chymases (α-chymases) share a common biochemical action in producing ANG II from angiotensin I and are predominant in tissue ANG II production (1, 26). In contrast, rats and mice also have β-chymases, which degrade ANG II. Therefore, hamsters are thought to be suitable animal models to evaluate the pathophysiological roles of
chymases and tissue RAS (8, 18, 20). Here, we showed that chymase-specific inhibitor TEI-E00548 partially, and TEI-F00806 completely, prevented proteinuria and renal mesangial expansion in diabetic hamsters. These findings suggest that chymase inhibition may be sufficient to protect against the progression of diabetic nephropathy. A number of experimental and clinical studies have shown that drugs targeting the RAS, including ANG II type 1 receptor blockers (ARB) and ACE inhibitors, offer some protective effects against diabetic nephropathy (3, 4, 32), which appear to be independent of their antihypertensive effects. However, the systemic components of the RAS are reported to be downregulated in diabetes (5, 40). Thus the intrarenal RAS is thought to be involved in the progression of diabetic nephropathy. The limited efficacy of ARB and ACE inhibitors may be due, at least in part, to insufficient suppression of the intrarenal RAS. Since the intrarenal ANG II concentrations are estimated to be much higher than those in plasma (27, 37), the effect of intrarenal ANG II may not be sufficiently blocked by conventional doses of ARB. As for the ACE inhibitor, our results showed that it completely suppressed the serum ANG II concentration with lowered systolic blood pressure, but did not attenuate the augmentation of intrarenal ANG II concentrations or did not have sufficient beneficial effects on renal dysfunction in diabetic hamsters. In contrast, chymase inhibitors completely suppressed the enhanced intrarenal ANG II production in diabetic hamsters without affecting serum ANG II levels or blood pressure. In addition, blockade of the RAS by ARB or the ACE inhibitor leads to a feedback-mediated increase in renin activity. The activated renin stimulates the conversion of ANG I and ultimately ANG II, which limits the efficacy of RAS inhibition. The increased renin level also acts through the prorenin/renin receptor, which may cause renal and cardiovascular injury (23). A renin inhibitor has recently become clinically available, and it alone or in combination with an ARB is expected to block the systemic RAS more efficiently than an ARB alone.
However, the effect of renin inhibitors on local ANG II production is still unknown. We confirmed that chymase inhibitors did not increase systemic renin activity in this study. Taken together, the present findings suggest that chymase inhibition most efficiently inhibits the intrarenal RAS in the diabetic kidney and may be a candidate for therapeutic intervention of diabetic nephropathy. Although ANG II might promote diabetic nephropathy through multiple pathways, it is well established that ANG II stimulates ROS production via the activation or increased expression of NAD(P)H oxidase in vascular cells. In kidneys, NOX4 is the main component of NAD(P)H oxidase (35). We have previously shown that the increased expression of NOX4 may play an important role in the increased production of ROS in diabetic renal tissues (9, 11). Gorin et al. (13) also reported that downregulation of NOX4 induced by antisense oligonucleotides attenuated oxidative stress, renal hypertrophy, and increased the renal expression of fibronectin in STZ-induced diabetic rats. In the present study, we showed that chymase-specific inhibitors attenuated oxidative stress and normalized the increased expression of NOX4 in diabetic renal tissues. The present results suggest that the protective effects of chymase inhibitors were at least partly mediated by normalization of the increased intrarenal ANG II levels and subsequent normalization of NOX4-derived ROS overproduction in diabetic kidneys.

Several in vitro studies have shown that high glucose levels or advanced glycation end products (AGEs) induce increases in intracellular chymase levels in podocytes, smooth muscle cells, and mesangial cells (6, 30, 46). However, because chymase is devoid of a signal peptide (36), it is unlikely that our immunohistochemical findings of chymase overexpression in renal tissues originated from intracellular chymase. Mast cells store abundant chymase in secretory granules. On release, chymase binds to the extracellular matrix and continues to function for several weeks (34). It is generally distributed in cardiovascular tissues along with the infiltration of chymase-positive inflammatory cells into the interstitium. Mast cells are present in human kidneys, and it is reported that their number increases in a variety of renal diseases, including diabetic nephropathy (41). Because low-grade inflammation is known to occur in diabetic vascular tissues, including renal tissues (7, 47), infiltrated inflammatory cells, such as mast cells, may be the source of the increased chymase levels. High glucose levels were reported to stimulate ROS production via protein kinase C-dependent activation of NAD(P)H oxidase (15, 17, 21, 22, 25). This high glucose effect may be the initiator of increased oxidative

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\text{(23, 39). However, the effect of renin inhibitors on local ANG II production is still unknown. We confirmed that chymase inhibitors did not increase systemic renin activity in this study. Taken together, the present findings suggest that chymase inhibition most efficiently inhibits the intrarenal RAS in the diabetic kidney and may be a candidate for therapeutic intervention of diabetic nephropathy. Although ANG II might promote diabetic nephropathy through multiple pathways, it is well established that ANG II stimulates ROS production via the activation or increased expression of NAD(P)H oxidase in vascular cells. In kidneys, NOX4 is the main component of NAD(P)H oxidase (35). We have previously shown that the increased expression of NOX4 may play an important role in the increased production of ROS in diabetic renal tissues (9, 11). Gorin et al. (13) also reported that downregulation of NOX4 induced by antisense oligonucleotides attenuated oxidative stress, renal hypertrophy, and increased the renal expression of fibronectin in STZ-induced diabetic rats. In the present study, we showed that chymase-specific inhibitors attenuated oxidative stress and normalized the increased expression of NOX4 in diabetic renal tissues. The present results suggest that the protective effects of chymase inhibitors were at least partly mediated by normalization of the increased intrarenal ANG II levels and subsequent normalization of NOX4-derived ROS overproduction in diabetic kidneys.}

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stress and inflammation, but the subsequent increases in chymase expression and local ANG II production may further accelerate ROS production and inflammation, ultimately forming a vicious cycle of oxidative stress and inflammation. This hypothesis should be evaluated in future studies.

In conclusion, this study showed for the first time that chymase-specific inhibitors protected against elevated intrarenal ANG II concentrations, oxidative stress, and renal dysfunction in diabetic hamsters. Since humans have α-chymase in common with hamsters and its upregulation in human diabetic renal tissues was reported, the present findings may be applicable to human diabetic nephropathy. The effectiveness of chymase-specific inhibitors should be further confirmed in human trials.

ACKNOWLEDGMENTS

The authors thank Teijin Pharma, Ltd., for kindly supplying TEI-E00548 and TEI-F00806. We also appreciate the technical support from the Research Support Center, Graduate School of Medical Sciences, Kyushu University.

GRANTS

This work was supported by a Grant-in-Aid for Scientific Research (16590888) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan, and by Special Coordination Funds for Promoting Science and Technology (SCF; funding program “Innovation Center for Medical Redox Navigation”).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
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