Meprin-α in chronic diabetic nephropathy: interaction with the renin-angiotensin axis

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Submitted 28 January 2005; accepted in final form 3 June 2005

Meprin-α (EC 3.4.24.18) is a metalloendopeptidase located in the brush-border membrane (BBM) of renal proximal tubule cells (3). Meprin-α is a metalloendopeptidase that is present in the renal proximal tubule brush-border membrane (BBM) and that colocalizes with angiotensin-converting enzyme (ACE). The MEPR gene locus on chromosome 18 has been linked to a heightened risk of diabetic nephropathy (DN) in patients with type 2 diabetes. This study evaluated 1) whether MEPR-α and MEPR-β gene and protein expression are altered in db/db mice before the onset of DN and 2) the role of MEPR-α in the pathogenesis of DN and the impact of the renin-angiotensin system on this interaction in two experimental models of diabetes. MEPR-α and MEPR-β gene and protein expression were evaluated in db/db mice, 13–14 wk of age, compared with lean C57BLKS/J littermate animals. A treatment study was then performed in which db/db mice and controls were assigned to one of three groups: control (C) water, no therapy; ACE inhibitor therapy, enalapril (EN)-treated water, 50 mg/l; and an ACE II protein type 1 blocker (ARB) therapy, losartan (LOS)-treated water, 500 mg/l. Treatment was started at 8 wk of age and continued for 52 wk. Male Sprague-Dawley rats with diabetes for 52 wk following a single dose of streptozocin (STZ; 60 mg/kg) were also studied. At 13.5 wk of age, MEPR-α and MEPR-β kidney mRNA abundance and protein expression were significantly lower in db/db mice compared with lean controls, with greater changes in MEPR-β (P < 0.05). In the treatment study, EN ameliorated and LOS exacerbated DN in db/db mice. BBM MEPR-α enzymatic activity and MEPR-α protein content were lower in db/db mice vs. control nonobese mice at 52 wk (P < 0.02). EN-treated db/db mice showed increased MEPR-α activity, MEPR-α content in BBM, decreased urinary MEPR-α excretion, and enhanced BBM staining for MEPR-α protein vs. C and LOS-treated db/db mice. In nonobese mice, EN and LOS treatment had no effect on MEPR-α expression. In rats with STZ-induced diabetes for 52 wk, urinary MEPR-α excretion was increased and MEPR-α activity and MEPR-α protein content per milligram of BBM protein were decreased compared with age-matched control animals (P < 0.05). These results indicate that db/db mice manifest decreased MEPR-α and MEPR-β gene and protein expression, before the development of overt kidney disease. Moreover, in db/db mice with DN and rats with STZ-diabetes, there was an inverse relationship between renal MEPR-α content and the severity of the renal injury. Treatment with an ACE inhibitor was more effective than ARB in ameliorating DN in db/db mice, a change that correlated with alterations in urinary excretion and BBM content of MEPR-α. MEPR-α may play a role in the pathogenesis of DN and the benefits of ACE inhibitor therapy on the progression of diabetic kidney disease may be related, in part, to its impact on renal MEPR-α expression.

Diabetic nephropathy (DN) is a leading cause of end-stage renal disease in the United States (14). The pathophysiology of this complication is still not fully understood. Current medical management of diabetes, consisting of tight glycemic control, aggressive management of blood pressure, and administration of angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARB), is effective in retarding the course of DN (8, 23). However, these treatments do not prevent DN and new therapeutic modalities are needed to care for patients with kidney involvement.

Meprins are metalloendopeptidases located in the brush-border membrane (BBM) of renal proximal tubule cells (3). Meprins are composed of two distinct subunits, α and β, that are encoded by distinct genes on human chromosomes 6 and 18, respectively (4). The mature meprin protein is an oligomer that is composed of varying combinations of α- and β-subunits. Any form of meprin that contains the meprin-α subunit is called meprin A (EC 3.4.24.18). The β-subunit retains a hydrophobic transmembrane domain, whereas this domain is removed by proteolytic processing of the meprin-α subunit (MEPR-α) during biosynthesis. Thus homooligomers of MEPR-α are secreted into the urine, while any combination of meprin subunits containing a β-subunit is anchored to the membrane (3).

Alterations in meprin A activity have been described in experimental models of kidney disease. The susceptibility to ischemic and nephrotoxic acute renal failure is decreased in inbred strains of mice with low meprin A activity (29). Meprin A activity in renal homogenates is altered in rats with streptozocin (STZ)-induced diabetes and puromycin aminonucleoside nephropathy (28). The role of meprin A in the pathogenesis of renal damage may depend on the acuity vs. chronicity of the disease process. Injury-induced alterations in cell-surface distribution of the metalloproteinase and alterations in membrane anchoring of meprin-α may be critical determinants of the impact of meprin A on the course of kidney disease. Thus, in acute renal injury, high levels of meprin A may be deleterious because of inappropriate localization of the enzyme in-d
ruptured tissue. In chronic forms of nephropathy, low meprin A activity may result in increased fibrosis and progression of glomerulopathies such as DN.

There is an increased susceptibility to develop nephropathy in patients with type 2 diabetes that is linked to chromosome 18, in the vicinity of the gene encoding the meprin-B subunit (15, 23a). Meprin-B is associated with ACE within the BBM of proximal tubule cells. In view of the role of the renin-angiotensin system (RAS) as a final common pathway mediating progressive DN, this raises the possibility that the beneficial effects of modulating the RAS in DN may be related, at least in part, to alteration in the expression and activity of membrane-bound meprin A in both type 1 and type 2 diabetes (3).

Therefore, we conducted the following studies to determine whether 1) MEP-α and MEP-β gene and protein expression are altered in db/db mice, a model of type 2 diabetes, before the onset of kidney disease; 2) there are changes in renal meprin A expression in chronic DN that develop in db/db mice; 3) long-term administration of an ACE inhibitor or ARB alters the course of DN in db/db mice; and 4) the effect of ACE inhibitor or ARB therapy on DN is related to the modulation of meprin A.

MATERIALS AND METHODS

Animals

Male C57BLKS/J-m +/+Lepr db/db and C57BLKS/J (lean, non-diabetic littermates) mice (Jackson Laboratories, Bar Harbor, ME) and male Sprague-Dawley rats (Taconic Farms, Germantown, NY) were housed in an animal facility that was maintained at 25°C with a 12:12-h light-dark cycle. Animals had free access to water and standard rodent chow (PMI Nutrition International, Brentwood, MO). The studies were approved by the Institutional Animal Care and Use Committee of Long Island Jewish Medical Center.

Disease Models

The following models of types 1 and 2 diabetes were studied.

**db/db Mice.** These animals, which lack the hypothalamic leptin receptor, represent a model of type 2 diabetes mellitus (10). They develop hyperglycemia by 4–7 wk of age with fasting serum glucose levels >250 mg/dl.

**STZ-diabetic Rats,** weighing 180–280 g at the onset of the experimental protocol, were given a single intraperitoneal injection of STZ, 60 mg/kg body wt (Upjohn, Kalamazoo, MI). This islet cell toxin induces a model of type 1 diabetes mellitus. STZ was dissolved in 0.01 M citrate buffer, pH 4. The diabetic state was confirmed by documentation of hyperglycemia, namely fasting serum glucose concentration >250 mg/dl, within 48 h after injection of STZ.

Experimental Design

The following two studies were performed.

**Initial.** db/db Mice (n = 9) and lean littersmate controls (n = 17) were killed at 13–14 wk of age to assess kidney size and renal expression of MEP-α and MEP-β genes and protein before the onset of clinically detectable DN.

**Treatment.** Twenty db/db mice were randomly assigned to one of the following three groups: 1) DB-C: mice given distilled water to drink, n = 6; 2) DB-EN: mice given drinking water containing the ACE inhibitor enalapril (EN), 50 mg/l, n = 7; and 3) DB-LOS: mice given drinking water containing the ARB losartan (LOS), 500 mg/l, n = 7. Twenty-two lean mice were randomized to the same three groups: 1) C-C: untreated drinking water, n = 7; 2) C-EN: EN-treated water, n = 8; and 3) C-LOS: LOS-treated water, n = 7.

Treatment was started at 8 wk of age when all of the db/db mice were hyperglycemic, and therapy was continued for 52 wk. A sample of mice (n = 3) from each group was killed after 24 wk and the remaining animals were maintained for the full 52-wk treatment period. The number of samples analyzed by the various experimental methods differed depending on the number of animals available in the specific groups at each study time point.

Urine samples and kidney tissue obtained from control, nondiabetic and untreated STZ-diabetic rats after 52 wk of observation were analyzed for urinary meprin-α excretion and renal meprin A enzymatic activity and BBM MEP-α content, respectively. These rats were investigated previously in an experimental protocol that was designed to evaluate the effect of antioxidant therapy on the course of chronic DN (27).

Sample Collection

**Urine.** Urine was obtained from db/db mice at the start of the study and at 4-wk intervals. Mice were placed in metabolic cages for 6–8 h and deprived of food but provided water ad libitum. Urine was centrifuged to remove debris and stored at −20°C.

**Serum.** Blood was collected from db/db mice at the start of the study and at 8-wk intervals. Animals were fasted for 24 h before blood sampling. Mice were anesthetized with CO₂ inhalation and blood was collected by puncture of the retro-orbital venous plexus. Blood was allowed to clot at room temperature, centrifuged at 13,000 g for 2 min, and the serum was stored at −20°C.

**Kidneys.** After 24 or 52 wk, mice were killed and both kidneys were rapidly removed. One intact kidney was snap-frozen at −70°C. The second kidney was cut in half longitudinally. One half was frozen and the other half was immersion fixed in 10% formalin.

Biochemical Analysis

Urine creatinine concentration was measured using a Beckman CX3 analyzer (Beckman Instruments, Brea, CA). Serum blood urea nitrogen (BUN), creatinine, triglycerides, total cholesterol, Na⁺, glucose, and albumin were determined with a Beckman CX3 Biochemistry analyzer.

Urinary MEP-α

Urinary MEP-α protein quantitation was determined according to the procedure of Beynon et al. (2, 9a). Briefly, urine samples were desalted using mini Quick Spin Oligo Columns (Boehringer Mannheim, Indianapolis, IN) that were prespun and preequilibrated with 50 μl of 20 mM HEPES, pH 7.6. Desalted urine samples, containing equivalent amounts of protein rather than equivalent amounts of creatinine, were subjected to immunoblotting for MEP-α protein detection. This approach was adopted because differences in meprin-α excretion are a consequence of alterations in proximal tubule structure and function rather than changes in glomerular filtration rate.

BBM Isolation

BBM were isolated from kidneys as previously described (28, 29). Briefly, one-quarter of a whole kidney was homogenized manually in 9 vol of a solution containing 2 mM Tris, pH 7, and 10 mM mannitol (homogenization buffer; Fujisawa USA, Deerfield, IL); 1 M MgCl₂ was added to achieve a final concentration of 10 mM MgCl₂. This mixture was stirred on ice for 15 min and then centrifuged at 1,500 g for 12 min. The sediment was discarded, and the supernatant was recentrifuged at 12,000 g for 12 min. Following the second centrifugation, the supernatant was discarded and the precipitate was suspended in 5 vol of homogenization buffer. These steps were repeated once more and the final precipitate was resuspended in 2 vol of homogenization buffer and stored at −70°C.

Renal Meprin A Activity in BBM

Fifty microliters of BBM samples and a blank solution were brought up to 125 μl with a buffer composed of 20 mM ethanolamine
in 0.14 M NaCl, pH 9.5, in a 1.5-ml Eppendorf microcentrifuge tube. One-hundred twenty-five microliters of a solution containing 22 mg/ml azocasein, prepared in the same buffer and prewarmed to 37°C, was added to samples. Tubes were vortexed and incubated with gentle agitation at 37°C for 30 min. The reaction was stopped by adding 1 ml of 5% trichloroacetic acid to each tube. The tubes were vortexed and centrifuged at 13,000 g for 4 min. One milliliter of supernatant was removed and absorbance was measured at 340 nm. Units of activity (U) were calculated as follows: (absorbance × 1,000)/min incubation, and the specific activity was normalized per milligram of BBM protein (28).

Western Immunoblot Analysis

Urine or BBM samples were reduced using a sample buffer containing 125 mM Tris-HCl, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, and 0.01% bromophenol blue. Reduced samples were run on a 7.5% polyacrylamide gel and then transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH). Following transfer, membranes were blocked and incubated with one of the following primary antibodies for 1 h: HMC-14 (a polyclonal rabbit antibody against mouse meprin-A), HMC-51/HMC-52 (polyclonal rabbit antibodies against mouse MEP-α), or PSU57 (polyclonal rabbit antibody against mouse MEP-β). Comparable results were obtained with the different anti-MEP-α antibody preparations. Following incubation with the primary antibody, membranes were washed with a solution containing 10 mM Tris, 100 mM NaCl, and 0.1% Tween 20, and then incubated for 1 h with a secondary antibody against rabbit IgG conjugated with horseradish peroxidase (Organon Technika, Buckinghamshire, UK), and radiographic films were developed. Densitometry was performed using an IS-1000 Digital Imaging System (Alpha Innotech, San Leandro, CA) to determine the relative intensities of the bands.

RNA Preparation

At age 13 wk, total RNA was extracted from the left kidneys of db/db and db/m mice (n = 8 for each group) using TRIZol (Invitrogen, Carlsbad, CA). The quantity and integrity of total RNA were determined by A260/A280 and agarose/formaldehyde gel electrophoresis, respectively.

Real-Time PCR

Synthesis of cDNA was performed with 2 μg of each RNA preparation, Superscript III Reverse transcriptase (Invitrogen) and hexanucleotide random primers (Roche, Indianapolis, IN). A reaction without reverse transcriptase was run in parallel for each RNA sample to control for DNA amplification. PCR Primers were designed with Primer express 1.5 software (Applied Biosystems) and the Spidey data to control for DNA amplification. PCR Primers were designed with Primer express 1.5 software (Applied Biosystems) and the Spidey data to control for DNA amplification. PCR Primers were designed with Primer express 1.5 software (Applied Biosystems) and the Spidey data to control for DNA amplification. PCR Primers were designed with Primer express 1.5 software (Applied Biosystems) and the Spidey data to control for DNA amplification. PCR Primers were designed with Primer express 1.5 software (Applied Biosystems) and the Spidey data to control for DNA amplification.

RESULTS

Initial Study

Male db/db mice and littermate controls were killed at 13–14 wk of age. At this point, the db/db mice weighed 33.7 ± 1.3 vs. 28.9 ± 0.3 g in littermate controls (P ≤ 0.05). Similarly, kidney weight was higher in db/db compared with nondiabetic lean mice, 0.236 ± 0.005 and 0.183 ± 0.003 g, respectively (P ≤ 0.05). In view of the parallel increases in total body and kidney weights, the kidney:body weight ratio was similar in the two groups of animals (data not shown). The relative abundance of MEP-α and MEP-β mRNA in the kidney was substantially decreased in the db/db mice compared with lean controls (Fig. 1). The changes in MEP-α and MEP-β gene

Histopathological Analysis

Routine pathology. Renal tissue was formalin fixed and thin sections were stained with hematoxylin-eosin and periodic acid-Schiff reagents. Segmental sclerosis was defined as localized collapse of capillary lumina and replacement with eosinophilic hyalinized material, occasionally accompanied by adhesions to Bowman’s capsule. Tubules and interstitium throughout the kidney were examined for dilatation, intratubular casts, tubular atrophy, interstitial infiltration with mononuclear cells, and replacement with fibrosis. The degree of tubulointerstitial alterations was graded on a scale from 0 to +3 as follows: 0, normal kidney; +1, mild abnormalities affecting <25% of the renal parenchyma; +2, moderate, 25–50% involvement; and +3, severe, >50% of the kidney involved. The pathologist (EV) was unaware of the group assignment of individual animals.

Immunohistochemical analysis. Paraffin-fixed renal tissue specimens were cut into 4- to 5-μm sections and placed onto polylysine-coated slides. They were air dried briefly and then baked overnight at 56°C. Sections were deparaffinized through graded alcohol and then rinsed in tap water. Relevant antigens were retrieved from sections by rapid heating with microwave irradiation (890 W) in the presence of citrate buffer for 15 min and then rinsed in tap water. Primary antibody was applied using a Ventana automated machine (Ventana Medical Systems, Tucson, AZ), and antibody detection was performed using a Ventana DAB detection kit. Slides were then counterstained in 2% Harris hematoxylin for 1 min, rinsed in tap water, and counterstained blue in 1.5% ammonia in 70% alcohol. Finally, slides were rinsed in tap water, dehydrated, and mounted.

Chemicals

All chemicals and reagents were purchased from Sigma (St. Louis, MO), unless otherwise stated. Losartan was provided by Merck (Rahway, NJ), and the anti-MEP-α and anti-MEP-β antibodies were produced in the laboratory of J. S. Bond, PhD.

Statistical Analysis

Results are expressed as means ± SE. To simplify the analysis of the db/db mice studies, some of the data were combined into early (0–8), middle (20–28), and concluding (44–52) phases of the 52-wk observation period. Differences between groups were compared using ANOVA and Student’s t-test with the Bonferroni correction for multiple comparisons. Relationships between variables were evaluated using a linear regression analysis. The mRNA expression data were assessed by Student’s t-test. Differences were considered significant if the P value was <0.05.

RESULTS

Initial Study

Male db/db mice and littermate controls were killed at 13–14 wk of age. At this point, the db/db mice weighed 33.7 ± 1.3 vs. 28.9 ± 0.3 g in littermate controls (P ≤ 0.05). Similarly, kidney weight was higher in db/db compared with nondiabetic lean mice, 0.236 ± 0.005 and 0.183 ± 0.003 g, respectively (P ≤ 0.05). In view of the parallel increases in total body and kidney weights, the kidney:body weight ratio was similar in the two groups of animals (data not shown). The relative abundance of MEP-α and MEP-β mRNA in the kidney was substantially decreased in the db/db mice compared with lean controls (Fig. 1). The changes in MEP-α and MEP-β gene
expression were paralleled by similar decreases in renal BBM meprin-α and meprin-β protein content (Fig. 2). The decrease in meprin subunit mRNA abundance and protein levels was greater for the β-chain vs. the α-chain. The amount of meprin-α protein in the BBM is dependent on the amount of meprin-β protein. The more dramatic decrease in meprin-α BBM protein than kidney meprin-α mRNA is most probably due to meprin-β decreases.

**Treatment Study**

Basic metabolic parameters and drug dosing. At the start of the experiment, db/db mice had a mean weight of 36.6 ± 2.2 g, whereas nondiabetic C57BLKS/J mice weighed 20.3 ± 1.4 g. Tables 1 and 2 summarize the growth, water intake, and urine output during the early (0–8 wk), middle (20–28 wk), and concluding (44–52 wk) phases of the study, for diabetic and control mice, respectively. In the db/db mice, there was a

### Table 1. Growth and water balance: db/db mice

<table>
<thead>
<tr>
<th></th>
<th>Early Phase</th>
<th>Middle Phase</th>
<th>Concluding Phase</th>
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<tbody>
<tr>
<td>Body wt, g</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>DB-C</td>
<td>47 ± 8</td>
<td>54 ± 12</td>
<td>44 ± 12</td>
</tr>
<tr>
<td>DB-EN</td>
<td>45 ± 7</td>
<td>50 ± 9</td>
<td>40 ± 10</td>
</tr>
<tr>
<td>DB-LOS</td>
<td>45 ± 8</td>
<td>50 ± 8</td>
<td>40 ± 9</td>
</tr>
<tr>
<td>Urine output, µl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DB-C</td>
<td>1,101 ± 390</td>
<td>1,139 ± 478</td>
<td>1,435 ± 462</td>
</tr>
<tr>
<td>DB-EN</td>
<td>1,063 ± 475</td>
<td>909 ± 318</td>
<td>1,233 ± 423</td>
</tr>
<tr>
<td>DB-LOS</td>
<td>747 ± 385</td>
<td>1,142 ± 560</td>
<td>1,489 ± 735</td>
</tr>
<tr>
<td>Water intake, ml/24 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DB-C</td>
<td>11.4 ± 1.3</td>
<td>16.5 ± 3.1*</td>
<td>8.8 ± 1.6</td>
</tr>
<tr>
<td>DB-EN</td>
<td>12.4 ± 2.2</td>
<td>18.8 ± 4.2*</td>
<td>10.0 ± 2.1</td>
</tr>
<tr>
<td>DB-LOS</td>
<td>8.9 ± 2.1</td>
<td>11.0 ± 1.4</td>
<td>8.2 ± 1.1</td>
</tr>
</tbody>
</table>

Data are provided as means ± SE. The early phase represents data obtained during weeks 0–8, the middle phase represents data obtained during weeks 20–28, and the concluding phase represents data obtained during weeks 44–52 of the 52-wk observation period. The number of mice in each group was: early phase, C, n = 6; enalapril (EN), n = 7; losartan (LOS), n = 7; middle phase, C, n = 6; EN, n = 7; LOS, n = 7; concluding phase, C, n = 3; EN, n = 4; LOS, n = 4. Enalapril-treated mice were given drinking water containing enalapril (50 mg/l) and losartan-treated animals received drinking water containing losartan (500 mg/l). *P < 0.05 vs. same animal group during the early and concluding phases and DB-LOS mice during all 3 phases of the study. DB-C, db/db mice, untreated, control; DB-EN, db/db mice, enalapril treated; DB-LOS, db/db mice, losartan treated.

### Table 2. Growth and water balance: littermate control db/m lean mice

<table>
<thead>
<tr>
<th></th>
<th>Early Phase</th>
<th>Middle Phase</th>
<th>Concluding Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-C</td>
<td>23 ± 2</td>
<td>27 ± 1*</td>
<td>28 ± 2*</td>
</tr>
<tr>
<td>C-EN</td>
<td>23 ± 2</td>
<td>27 ± 2*</td>
<td>28 ± 2*</td>
</tr>
<tr>
<td>C-LOS</td>
<td>23 ± 3</td>
<td>27 ± 1*</td>
<td>28 ± 1*</td>
</tr>
<tr>
<td>Urine output, µl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-C</td>
<td>487 ± 286</td>
<td>201 ± 134</td>
<td>257 ± 151</td>
</tr>
<tr>
<td>C-EN</td>
<td>453 ± 387</td>
<td>311 ± 307</td>
<td>367 ± 196</td>
</tr>
<tr>
<td>C-LOS</td>
<td>222 ± 113</td>
<td>403 ± 368</td>
<td>326 ± 206</td>
</tr>
<tr>
<td>Water intake, ml/24 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-C</td>
<td>5.6 ± 0.9</td>
<td>5.5 ± 0.8</td>
<td>5.1 ± 0.8</td>
</tr>
<tr>
<td>C-EN</td>
<td>6.6 ± 1.5</td>
<td>5.3 ± 1.1</td>
<td>5.1 ± 1.1</td>
</tr>
<tr>
<td>C-LOS</td>
<td>5.7 ± 0.3</td>
<td>5.5 ± 0.8</td>
<td>5.0 ± 0.3</td>
</tr>
</tbody>
</table>

Data are provided as means ± SE. The early phase represents data obtained during weeks 0–8, the middle phase represents data obtained during weeks 20–28, and the concluding phase represents data obtained during weeks 44–52 of the 52-wk observation period. The number of mice in each group was early phase, C, n = 7; EN, n = 8; LOS, n = 7; middle phase, C, n = 7; EN, n = 7; LOS, n = 7; and concluding phase, C, n = 4; EN, n = 4; LOS, n = 4. Enalapril-treated mice were given drinking water containing enalapril (50 mg/l) and losartan-treated animals received drinking water containing losartan (500 mg/l). *P < 0.05 vs. comparable group during the early phase. C-C, littermate, nondiabetic mice, untreated; C-EN, littermate, nondiabetic mice, enalapril treated; C-LOS, littermate, nondiabetic mice, losartan treated.
decline in body weight from the midpoint until the end of the study that was paralleled by decreased water intake. These changes were not significant because of the small number of animals maintained for the entire 52-wk observation period. Urine output increased slightly over the course of the study without any significant intergroup differences. Nondiabetic mice steadily increased their body weight over the 52-wk period (P < 0.05), and their water intake and urine output were fairly constant. Survival was 100% in both strains, except for one DB-C mouse that died after 51 wk of observation.

During the 52-wk treatment period, based on the average water intake, in the db/db mice the daily EN dose ranged from 12.7 to 18.7 mg/kg body wt and the LOS dosage was 100–110 mg/kg body wt. In the lean nondiabetic animals, the estimated daily EN and LOS dosages were 9.3–14.5 and 90–127 mg/kg body wt, respectively.

Serum biochemical analysis. Among the db/db mice, there were no significant intergroup differences in serum glucose, creatinine, sodium, cholesterol, or triglycerides concentrations over the course of the experiment (Table 3). However, BUN concentration was higher in DB-LOS mice compared with DB-C or DB-EN animals. This difference was first noted at 8–16 wk of treatment and persisted until the end of the experiment (P < 0.001; Table 3). Although water intake in the DB-LOS group was lower than in the other two groups during the middle phase of treatment, it was comparable during the concluding period, indicating that dehydration was not the cause of the elevated BUN in DB-LOS mice. Among the nondiabetic mice groups, there were no significant differences in serum glucose, creatinine, sodium, cholesterol, triglycerides, or BUN concentrations over the course of the experiment (data not shown).

Urinary MEP-α excretion. Urinary MEP-α excretion was higher in db/db compared with nondiabetic mice at 24 and 52 wk (Fig. 3, A and B, respectively). The overall difference between the strains was statistically significant at the earlier time point (P < 0.0005); however, this was not the case after 52 wk of treatment because of the small sample size (n = 3 in each group) and increased urinary MEP-α excretion with aging in control animals. There were no consistent intergroup differences between C, EN, and LOS-treated diabetic animals at either of the two time points examined.

Renal meprin A activity. After 52 wk of diabetes, meprin A enzymatic activity of BBM was lower in all db/db mice compared with lean controls (P < 0.005; Table 4). Although the difference between the two strains was not significant in the untreated mice because of the small number of db/db mice (n = 3), the decrease in meprin A activity was significant in diabetic mice that received ACE inhibitor or ARB treatment (P < 0.05).

Immunoblot analysis of BBM proteins for MEP-α. At 8-wk age, before treatment, there was no difference in BBM MEP-α content in db/db and nondiabetic mice (compare with Initial Study in 13- to 14-wk-old mice). However, after 52 wk, MEP-α protein was lower in the BBM of db/db mice than in nondiabetic animals (P < 0.01; Fig. 4A and Table 5). Administration of EN enhanced MEP-α content in BBM compared with D-C and D-LOS mice (P ≤ 0.01) to levels that were similar to those noted in C-C and C-EN mice (P ≥ 0.3). Treatment with LOS had no effect on MEP-α expression in db/db mice. Nondiabetic mice did not display any variation in MEP-α expression in response to ACE inhibitor or ARB treatment.

Renal immunohistochemical staining for MEP-α. Immunohistochemical staining for MEP-α, which was localized to the proximal tubule BBM, was decreased in db/db mice compared with control mice. At 24 wk, there was no demonstrable difference between the three db/db groups with regard to the intensity of staining for MEP-α. However, by 52 wk, there was more intense staining in proximal tubules of DB-EN mice than in DB-C and DB-LOS animals (Fig. 5). The amount of staining in DB-EN kidneys was comparable to that observed in nondiabetic kidneys, whereas it was lower in DB-C and DB-LOS mice. There was no variation in renal BBM staining for MEP-α in response to ACE inhibitor or ARB therapy in the three groups of lean mice.

Renal histopathology. There were minor renal histopathological changes at 24 wk in db/db mice that were slightly more prominent in the DB-LOS group vs. DB-C and DB-EN. However, after 52 wk, renal histopathological changes were clearly evident in db/db mice. The extent of glomerular and tubular pathology was more severe in DB-LOS kidneys vs. DB-C and DB-EN kidneys. DB-LOS mice displayed more mesangial hypercellularity and glomerular sclerosis than the other diabetic mice, although the intragroup differences were not significant. The extent of tubular injury, based on the number of dilated tubules and intratubular casts, was markedly higher in the DB-LOS mice (P < 0.01; Table 6). There was no renal

Table 3. Serum biochemical values: db/db mice

| Glucose, mg/dl | Control Early | 183±90 | 213±95 | 207±60 |
| Creatinine, mg/dl | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 |
| Sodium, mg/dl | 158±3 | 157±11 | 159±6 | 157±2 |
| Cholesterol, mg/dl | 94±9 | 104±13 | 90±9 | 90±10 |
| Triglycerides | 97±15 | 112±24 | 92±17 | 97±13 |
| BUN, mg/dl | 25±6 | 26±10 | 23±6 | 34±11 |
| Albumin, mg/dl | 2.6±0.1 | 2.6±0.1 | 2.3±0.1 | 2.5±0.1 |

Data are reported as means ± SE. The early phase represents data obtained during weeks 0–8, the middle phase represents data obtained during weeks 20–28, and the concluding phase represents data obtained during weeks 44–52 of the 52-wk observation period. The number of mice in each group was early phase, C, n = 6; EN, n = 7; LOS, n = 7; middle phase, C, n = 6; EN, n = 7; LOS, n = 7; concluding phase, C, n = 3; EN, n = 4; LOS, n = 4. Enalapril-treated mice were given drinking water containing enalapril (50 mg/l) and losartan-treated animals received drinking water containing losartan (500 mg/l). *P < 0.05 vs. all other groups.
pathology in any of the three groups of lean mice. Examples of the renal histopathology in an untreated control mouse and the three groups of \textit{db/db} mice are provided in Fig. 6.

**STZ-Diabetes**

Urinary MEPRIN-\alpha levels were increased in STZ-diabetic vs. nondiabetic rats at 52 wk (\(P < 0.05\); Fig. 3C). In addition, in rats with STZ-diabetes for 52 wk, meprin A enzymatic activity in BBM was lower in diabetic animals compared with age-matched controls, 36 ± 10 (\(n = 3\)) vs. 110 ± 31 (\(n = 4\)) U/mg protein, respectively (\(P = 0.05\)). Finally, STZ-diabetic rats also had decreased MEPRIN-\alpha content in the BBM vs. nondiabetic rats after 52 wk of observation (\(P < 0.025\); Fig. 4B).

**DISCUSSION**

Previous experimental studies suggested that meprin A activity and expression might affect renal structure and function in various disease states such as acute ischemic and nephro-

**Fig. 3.** Western immunoblot analysis of urinary MEPRIN-\alpha excretion. Urine samples containing equivalent amounts of protein were incubated with HMC-51/ HMC-52 (polyclonal) antibody to meprin-\alpha, respectively, and secondary antibody for visualization. A urine sample was analyzed from all animals in each experimental group and these are representative immunoblots. Enalapril (EN)-treated mice were given drinking water containing enalapril (50 mg/l) and losartan (LOS)-treated animals received drinking water containing losartan (500 mg/l). Lane markers: CON, positive, control; DB-C, \textit{db/db} mice, untreated; DB-EN, \textit{db/db} mice, enalapril treated; DB-LOS, \textit{db/db} mice, losartan treated; C-C, littermate, nondiabetic mice, untreated; C-EN, littermate, nondiabetic mice, enalapril treated; C-LOS, littermate, nondiabetic mice, losartan treated; C, normal rat; STZ, streptozocin-diabetic rat. A: \textit{db/db} mice after 24 wk. The attached graph summarizes the densitometry readings in arbitrary units, reported as means ± SE, \(n = 3\) for each condition. \(*P < 0.0005\) \textit{db/db} vs. nondiabetic mice. B: \textit{db/db} mice after 52 wk. The attached graph summarizes the densitometry readings in arbitrary units, \(n = 3\) for each condition. \(*P \leq 0.05\) vs. control nondiabetic condition. C: STZ-diabetic rats after 52 wk. The attached graph summarizes the densitometry readings in arbitrary units, \(n = 4\) for each condition. \(*P < 0.05\) vs. control nondiabetic condition.
toxic renal failure, unilateral ureteral obstruction, and chronic puromycin aminonucleoside nephropathy (24, 28, 29). Recent data suggest a linkage between the gene for MEP-β and susceptibility to nephropathy in type 2 diabetes (23a). Therefore, in this study, we focused on this association in experimental models of diabetes. Our findings suggest that meprin A plays a role in the development of the chronic nephropathy in STZ-treated rats and db/db mice, models of type 1 and type 2 diabetes, respectively. None of the available experimental models of diabetes fully reproduce the nephropathy observed in patients with this disease. However, based on the histological alterations in the mesangium and tubulointerstitium, the models used in these studies are widely considered relevant to the chronic renal disease observed in clinical diabetes.

The pathogenesis of chronic DN is multifactorial (8, 14). Inflammatory mediators such as monocyte chemoattractant protein-1 and osteopontin, peptide growth factors including transforming growth factor (TGF)-β and vascular endothelial growth factor, and hormones such as ANG II are implicated in this process (11, 14, 32). The findings in this report indicate that meprin A in conjunction with the RAS is also involved. In db/db mice, changes in MEP-α and MEP-β gene and protein expression preceded the onset of overt DN. Moreover, in both db/db mice and rats with STZ-diabetes, renal meprin A activity and MEP-α expression and content in BBM were diminished and were inversely related to the extent of renal injury. If diabetes is considered a condition of accelerated aging (31), then the kidney injury associated with chronic DN may be a consequence of an exaggerated decline in renal meprin A activity. In db/db mice, long-term administration of ARB further decreased MEP-α expression and exacerbated renal injury. In contrast, ACE inhibitor treatment attenuated DN and enhanced MEP-α expression. Confirmation of a role for meprin A in DN will require further studies using well-tolerated selective inhibitors of the enzyme or animals that have been genetically manipulated to overexpress or delete the protein.

In view of the changes in both MEP-α and MEP-β observed in the initial study, it is important to explain why the experimental protocol focused on MEP-α in the treatment study. First, well-characterized antibodies were only available against MEP-α at the time of these investigations. Second, it enabled correlations to be made between changes in renal parenchymal expression and urinary excretion of the α-subunit. This would not apply to MEP-β, which is membrane bound and not present in the urine. The parallel changes in MEP-α and MEP-β at 13–14 wk suggest that there is a linkage between the two meprin subunits in the pathogenesis of DN. This suppression is buttressed by the decrease in meprin A enzymatic activity, which requires both subunits.

In the treatment study, food and water intake were assessed weekly. Although drug doses were not calculated on a daily basis and animals were not pair fed, water intake and weight gain in each group were fairly steady over the course of the study. This suggests a corresponding stability in dosing of the two test drugs during the 52-wk treatment period. Lack of 24-h urine collections precluded the use of creatinine clearance as a

Data are reported as means ± SE and represent meprin enzymatic activity per mg brush-border membrane (BBM). The values in parentheses indicate the number of mice in each experimental group. Enalapril-treated mice were given drinking water containing enalapril (50 mg/l) and the losartan-treated animals received drinking water containing losartan (500 mg/l). *P < 0.005 db/db vs. nondiabetic mice (ANOVA). †P < 0.05 db/db vs. similarly treated nondiabetic mice.

<table>
<thead>
<tr>
<th>Control</th>
<th>Enalapril treated</th>
<th>Losartan treated</th>
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</thead>
<tbody>
<tr>
<td>80 ± 38 (3)</td>
<td>61 ± 13 (4)†</td>
<td>47 ± 9 (4)†</td>
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<table>
<thead>
<tr>
<th>Littermate, Nondiabetic Mice</th>
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<tr>
<td>147 ± 41 (4)</td>
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**Table 4.** Meprin A activity in isolated BBM

**Fig. 4.** Western immunoblot analysis of BBM MEP-α. Equivalent amounts of BBM protein were subjected to immunoblotting using the same methods described for urine meprin-α excretion. A: representative immunoblots of db/db mice after 52 wk of treatment. The attached graph summarizes the densitometry readings in arbitrary units, n = 3 for each condition. *P ≤ 0.005 vs. paired control condition. **P ≤ 0.01 vs. other 2 diabetic conditions. B: STZ-diabetic rats after 52 wk. The attached graph summarizes the densitometry readings in arbitrary units, n = 4 for each condition. *P ≤ 0.025 vs. control nondiabetic condition.
marker of renal function and BUN and serum creatinine concentration and histopathological alterations were utilized as primary evidence of progressive renal disease. Albuminuria was not measured for the following reasons: 1) a dissociation between albumin excretion and histopathological severity of DN has been observed in db/db mice treated with anti-TGF-β antibody (34) and 2) chronic experimental diabetes results in urinary excretion of molecular forms of albumin that do not cross react with antibodies used in commercially available assay kits (12).

Blood pressure, plasma renin activity, and plasma aldosterone levels were not measured as part of this study because of technical constraints and the limited availability of plasma from the mice with chronic DN. We suggest that failure to measure blood pressure does not detract from the main conclusions for the following reasons: 1) the doses of the study drugs, EN and LOS, match previous reports in experimental DN and the efficacy is likely to match these earlier studies; 2) experimental DN is not characterized by severe hypertension; 3) intermittent measurements of blood pressure are less critical for analysis of drug effect in a chronic treatment period of 52 wk in which blood pressure is likely to fluctuate widely; and 4) meprin A expression and activity are not known to be affected directly by alterations in blood pressure per se.

### Table 5. Renal BBM meprin-α content in db/db mice: time course

<table>
<thead>
<tr>
<th>Treatment Week</th>
<th>Renal BBM Meprin-α Content DB-C Mice, %C-C mice</th>
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<tbody>
<tr>
<td>0</td>
<td>78%</td>
</tr>
<tr>
<td>24</td>
<td>62%</td>
</tr>
<tr>
<td>52</td>
<td>41%*</td>
</tr>
</tbody>
</table>

BBM meprin-α content in equivalent amounts of protein was determined by immunoblot analysis. The values represent mean densitometry readings for meprin-α with 3 samples at each time point. *P < 0.01 vs. 0 and 24 wk.

### Table 6. Renal histopathology: 52 wk

<table>
<thead>
<tr>
<th>Animal/Group</th>
<th>Glomerular Injury Score</th>
<th>Tubulointerstitial Injury Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB-C (n = 3)</td>
<td>1.0±0.6</td>
<td>0</td>
</tr>
<tr>
<td>DB-EN (n = 4)</td>
<td>1.3±0.5</td>
<td>0</td>
</tr>
<tr>
<td>DB-LOS (n = 4)</td>
<td>2.3±0.3</td>
<td>1.6±0.4*</td>
</tr>
</tbody>
</table>

Data are reported as means ± SE. The renal histopathological abnormalities were graded on a scale from 0 to 3+ as follows: 0, normal kidney; 1+, <25% of kidney affected; 2+, 25–50% of kidney affected; 3+, >50% of renal parenchyma affected. The numbers in parentheses indicate the number of mice in each group. *P < 0.01 vs. tubulointerstitial injury scores in DB-C and DB-EN groups.

Fig. 5. Immunohistochemistry of MEP-α in renal cortex. Paraffin-fixed tissue was deparaffinized through graded alcohol. Sections were rapidly heated with microwave irradiation (890 W) in citrate buffer for 15 min. Primary antibody was applied and detected using Ventana DAB kit. Slides were counterstained with 2% hematoxylin and counterstained blue with 1.5% ammonia in 70% alcohol. The photomicrographs are ×50 magnification. A: control mouse, no treatment (C-C) after 52 wk. B: db/db mouse, no treatment (DB-C) after 52 wk. C: db/db mouse, enalapril treatment (DB-EN) after 52 wk. D: db/db mouse, losartan treatment (DB-LOS) after 52 wk.
There are several important findings that emerged from these studies. First, renal meprin A enzymatic activity and BBM protein content of MEP-α/H9251 were influenced by the diabetic state per se rather than genetically determined variability in meprin A enzyme activity (9). Chronic hyperglycemia in db/db mice resulted in decreased enzymatic activity and decreased amounts of tissue-bound MEP-α in the BBM and diminished immunohistochemical staining for meprin-α protein at 24 and 52 wk. The same pattern was also observed in untreated STZ-diabetic rats after 52 wk. The findings in the 13- to 14-wk-old db/db mice suggest that altered renal MEP-α expression and meprin A activity are secondary to reduced meprin-α and meprin-β gene transcription and protein synthesis. Further studies are needed to exclude abnormal meprin subunit mRNA stability or translation, or processing of newly synthesized protein subunits in DN.

Second, MEP-α excretion in the urine was increased in diabetic vs. normoglycemic animals, in both models of diabetes. This abnormality was manifest in db/db mice at 24 wk, before detectable renal histopathological abnormalities. There was an inverse relationship between tissue MEP-α content and meprin A proteolytic activity on the one hand and urinary excretion of MEP-α. By 14 wk of age, meprin-β gene expression was decreased >75% in db/db mice relative to levels in their lean littermates, while meprin-α had decreased 28%.

Because meprin-α depends on meprin-β to attach to the brush border, meprin-α on the brush border would be expected to decrease along with meprin-β in agreement with our observations. Increasing the meprin-α to meprin-β ratio above a certain value will lead to a greater proportion of total meprin-α that will be excreted in urine of the db/db mice. The greater proportion of total meprin-α secreted, combined with only a slight decrease in total meprin-α gene expression, accounts for the observed net increase in meprin-α excretion.

Microalbuminuria has been used as a clinical marker of DN (13, 20). MEP-α level may also be elevated in the urine of patients with type 1 and/or type 2 diabetes in the early stages of the disease as well as after the development of overt DN (21). The data from the 13- to 14-wk-old db/db mice indicate that disturbances in meprin subunit metabolism and excretion precede the onset of clinically evident DN. Clinical studies are needed to evaluate the predictive role of urinary MEP-α excretion compared with standard indexes such as microalbuminuria in detecting incipient DN (6).

Third, our findings confirm the renoprotective effect of ACE inhibitor therapy against the progression of DN in db/db mice (8, 14, 23). In contrast, ARB therapy with LOS did not protect against DN. The EN and LOS doses were comparable to those used in previous studies. Dose-response studies were not performed because of the high cost and the
difficulty in maintaining animals with chronic untreated diabetes. However, the difference in efficacy of EN vs. LOS was observed in two separate protocols and it was associated with divergent effects of the two drugs on meprin A activity and MEP-\(\alpha\) expression in \(db/db\) mice. Treatment with an ACE inhibitor or ARB amplified the inverse relationship between meprin A enzymatic activity and MEP-\(\alpha\) content in the renal BBM and the severity of DN but in opposite directions. Moreover, use of these two drugs was associated with alterations in urinary MEP-\(\alpha\) excretion that paralleled the degree of kidney damage.

It is important to emphasize that a 1-yr treatment period is highly unusual in studies of DN. In a recent review of nephropathy in \(db/db\) mice, none of the cited investigations about renal function or histopathology involved observation periods longer than 25 wk (26). The extended 52-wk treatment phase in this report is unique for this model of diabetes and enables valuable inferences to be made vis-à-vis human disease, which develops after a lengthy latency period. Our findings suggest that in chronic DN, it may be harmful to block the angiotensin type 1 (AT-1) receptor with agents such as LOS, while leaving the type 2 receptor unoccupied (6). The beneficial effect of ACE inhibitors in slowing the progression of DN may involve reduction in the circulating levels of ANG II and potentiation of intrarenal bradykinin levels (30).

We propose the following scheme to explain the observations in this report (Fig. 7). As a first step, ANG II, which is increased in diabetes, downregulates MEP-\(\alpha\) synthesis and reduces enzyme expression in the kidney via the AT\(_2\) receptor. Lower levels of MEP-\(\beta\) in renal BBM result in decreased anchoring of MEP-\(\alpha\) to the apical membrane and lower meprin A enzymatic activity. This would lead to higher urinary excretion of MEP-\(\alpha\) in diabetic animals. In addition, it would be consistent with the linkage between polymorphism in the MEP-\(\beta\) gene and susceptibility to nephropathy in type 2 diabetes. ACE inhibition leads to upregulation of MEP-\(\beta\), which, in turn, results in the normalization of MEP-\(\alpha\) content in the BBM and urinary MEP-\(\alpha\) excretion. Changes in the metabolism of the meprin subunits may contribute to the development of DN by two distinct mechanisms. First, alterations in meprin A activity may modify the proteolysis of profibrotic agents such as TGF-\(\beta\) and osteopontin (11, 14). Decreased meprin A activity in diabetes may also exacerbate nephropathy by interfering with digestion of extracellular matrix proteins such as laminin and nidogen (16, 33), leading to glomerulosclerosis and tubulointerstitial fibrosis. Second, increased urinary excretion of MEP-\(\alpha\) may directly injure renal tubule cells and promote tubulointerstitial fibrosis in DN. This is supported by the demonstration of MEP-\(\alpha\)-induced cytotoxicity to renal tubular epithelial cells (LLC-PK\(_1\) and Madin-Darby canine kidney cells) in vitro (7).

**GRANTS**

This work was supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Grants RO1-DK-54625 and RO1-DK-19691 (to J. S. Bond).
REFERENCES


