Upregulation of soluble epoxide hydrolase in proximal tubular cells mediated proteinuria-induced renal damage

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1Department of Nephrology, Peking University Third Hospital, Beijing, China; 2Department of Physiology and Pathophysiology, Peking University Health Science Center, Key Laboratory of Molecular Cardiovascular Science of the Ministry of Education, Beijing, China; 3Department of Geriatric Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan Province, China; and 4Department of Entomology and Cancer Center, University of California, Davis, California

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Wang Q, Pang W, Cui Z, Shi J, Liu Y, Liu B, Zhou Y, Guan Y, Hammock BD, Wang Y, Zhu Y. Upregulation of soluble epoxide hydrolase in proximal tubular cells mediated proteinuria-induced renal damage. Am J Physiol Renal Physiol 304: F168–F176, 2013. First published November 14, 2012; doi:10.1152/ajprenal.00129.2012.—Soluble epoxide hydrolase (sEH) converts EETs into their corresponding less biologically active molecules. The regulation of sEH in the kidney was positively correlated with proteinuria and negatively with serum albumin level. To investigate the role of sEH in proteinuria-induced renal damage, we incubated purified urine protein from patients with renal proximal tubular epithelial cells in vitro. The level of sEH in the cortex was elevated, as were monocyte chemoattractant protein 1 and the epithelial-mesenchymal transition of sEH in human kidney by examining biopsies from 153 patients with a variety of glomerulonephritis, including minimal-change, membranous, and IgA nephropathy. Immunohistochemical staining of frozen kidney biopsy samples revealed sEH preferentially expressed in the renal proximal tubular cells, and its expression increased in all patients with glomerulonephritis. The level of sEH in the cortex was positively correlated with proteinuria and negatively with serum albumin level. To investigate the role of sEH in proteinuria-induced renal damage, we incubated purified urine protein from patients with renal proximal tubular epithelial cells in vitro. The level of sEH in the cortex was positively correlated with proteinuria and negatively with serum albumin level. To investigate the role of sEH in proteinuria-induced renal damage, we incubated purified urine protein from patients with renal proximal tubular epithelial cells in vitro. The level of sEH in the cortex was positively correlated with proteinuria and negatively with serum albumin level.

EPOXYEICOSATRIENOIC ACIDS (EETs) are cytochrome P-450 (CYP) metabolites of arachidonic acid (AA) with potent biological effects, such as vasorelaxation, promotion of sodium excretion and profibrinolytic activities, as well as anti-inflammatory and antiproliferative effects (3, 9, 19, 31). Several regioisomeric EETs (5,6-, 8,9-, 11,12-, 14,15-) have been implicated as endothelium-derived hyperpolarizing factors and potent dilate numerous vascular beds through their activation of calcium-dependent K+ (KCa) channels in vascular smooth muscle cells (3, 31, 40). EETs may prevent vascular inflammation by a mechanism involving inhibition of adhesion molecule expression and modulate the growth of vascular smooth muscle cells in vitro and in vivo (9, 19, 32). Soluble epoxide hydrolase (sEH) converts EETs into their corresponding less biologically active molecules. The activity of sEH is therefore thought to be a major determinant of EET bioavailability (6, 14). Genetic deletion of sEH, as well as pharmacological inhibition, increases plasma EET levels and potentiates their effects (22, 23); thus sEH inhibition has antihypertensive and anti-inflammatory effects. Indeed, sEH inhibition may reduce hypertension, myocardial infarct size, cerebral infarction, vascular remodeling and atherosclerosis, and even tobacco smoke-induced airway inflammation (5, 13, 30).

The kidney produces all four regioisomeric EETs (32), and both EET-related CYPs (2C8, 2C9, and 2J2 in human) and sEH inhibition is expressed in the kidney at high levels (2). One important progression factor of chronic renal disease is hypertension. The antihypertensive effect of sEH inhibition has been demonstrated in numerous rat and mouse models, such as spontaneously hypertensive rats, angiotensin II (AngII)-induced hypertension, and deoxycorticosterone acetate-salt-induced and salt-sensitive hypertension (2, 15, 16, 20, 25, 39). The antihypertensive effect was believed to be mediated by decreased vascular resistance and enhanced renal Na+ excretion, which resemble biological effects of EETs.

Beyond vasodilatation, EETs also function as endogenous anti-inflammatory, antiproliferative agents that might protect blood vessels against inflammation and sclerosis (6, 21). Glomerular inflammation and sclerosis are primary pathological features of glomerulonephritis, and proteinuria is one of the main symptoms and a major progression factor for glomerulonephritis. EETs have effects on renal blood flow, glomerular filtration rate, and urinary sodium excretion rate (12, 29). sEH inhibition protects the kidney against inflammatory components of hypertension by attenuating glomerular macrophage filtration rate, diminution of mesangial proliferation, and renal arteriolar intimal thickening (25). Thus it is logical to assume that sEH inhibition is a strategy to prevent progression of glomerulonephritis (12). Accordingly, sEH inhibition was reported to at-
and transferrin, were from Sigma (St. Louis, MO). Antibodies against including recombinant human epidermal growth factor (EGF), insulin, serum (FBS) was from Hyclone. All other cell culture supplements, provided by Drs. Paul D. Jones and Bruce D. Hammock (University of California, Davis), as described (2, 34). The adenoviral vector encoding full-length human sEH (Ad-sEH) was created in our laboratory as described earlier. sEH selective inhibitor 1-(1-methylsulfonyl-2-phenyl-1H-benzimidazol-5-yl)ethyl (H9251) is a consequence of glomerulosclerosis and interstitial fibrosis. Relative ly is known about CYP-catalyzed AA metabolism and the biological effects of the resulting eicosanoids in human kidney. sEH expression and cellular localization in kidney, as well as the role of sEH in glomerulonephritis, especially the relation with proteinuria, hypertension, and renal function, is not well studied. In this study, we investigated the renal expression of sEH in various forms of glomerulonephritis in humans and the relation of sEH and clinical characteristics. sEH was upregulated in proximal tubular cells in patients with glomerulonephritis, which was associated with proteinuria but not hypertension. Furthermore, we studied the role of the sEH in proteinuria-induced cell injury in rat proximal tubular epithelial cells (RPTCs) in vitro and in an adriamycin (ADR)-induced nephropathy mouse model in vivo. The upregulation of sEH in proximal tubular cells in chronic proteinuric kidney diseases may mediate the proteinuria-induced renal damage; sEH inhibition by increasing renal eicosanoids levels could be translated to therapies for preventing the progression of chronic proteinuric kidney diseases to end-organ damage.

**MATERIALS AND METHODS**

**Patients.** All procedures and use of anonymous tissue were according to guidelines of the ethics committee of Peking University Third Hospital. We collected renal biopsy specimens and clinical data from 153 inpatients who were admitted to the Nephrology Department of Peking University Third Hospital from May 2008 to March 2009. All patients gave informed consent to be in this study. The pathological diagnoses were minimal change nephropathy (MCD, n = 28), membranous nephropathy (MN, n = 50), and IgA nephropathy (IgAN, n = 75). Renal normal tissue specimens from the cortex apart from tumor tissue of patients with renal carcinoma (n = 10, age and sex matched) served as controls. Clinical parameters such as edema, blood pressure, serum albumin level, 24-h proteinuria, and serum creatinine level were measured 2 to 4 days before biopsy.

**Reagents.** DMEM/F12 medium was from GIBCO, and fetal bovine serum (FBS) was from Hyclone. All other cell culture supplements, including recombinant human epidermal growth factor (EGF), insulin, and transferrin, were from Sigma (St. Louis, MO). Antibodies against sEH, CYP2C9, Tamm-Horsfall protein (THP), β-actin, α-smooth muscle actin (α-SMA), and GAPDH were from Sigma, Cayman, and Abcam, respectively. sEH selective inhibitor 1-(1-methylsulfonyl-piperidin-4-yl)-3-(4-fluoromethoxy-phenyl)-urea (TUPS) was kindly provided by Drs. Paul D. Jones and Bruce D. Hammock (University of California, Davis), as described (2, 34). The adenoviral vector encoding full-length human sEH (Ad-sEH) was created in our laboratory as previously reported (2). Polyvinyl B-immobilized columns for removal of endotoxin were from Detoxi-Gel (Pierce Chemical, Rockford, IL).

**Immunohistochemistry.** Frozen slices of biopsy sections from patients were heat pretreated and blocked with 10% goat serum in PBS, then incubated with primary antibody, including anti-sEH (1:100), anti-CYP2C9 (1:100), or anti-THP and horseradish peroxidase-conjugated secondary antibody, then counterstained with diaminobenzidine tetrahydrochloride, dehydrated, and mounted. The staining of sEH or CYP2C9 was scored semiquantitatively by estimating the percentage of cortical tubules expressing the corresponding protein per field. The staining was presented as + for 0–25%; ++ for 25–50%; +++ for 50–75%, and ++++ for 75–100%. Immunohistochemistry staining was scored blindly by two nephropathologists, independently.

**Extraction of urinary protein.** Three urine samples from patients with MCD, MN, or IgAN, who had not received glucocorticoid or immunosuppressive treatment, were collected and pooled. Urinary proteins were isolated and purified by ammonium sulfate precipitation. The protein components were analyzed by 10% SDS-PAGE and Coomassie blue staining (26). The extracted protein liquid was freeze-dried to protein powder. Before being added to cell cultures, powder was resolved by culture medium, endotoxin was removed by use of polymyxin B-immobilized columns, and powder was filtered through a 0.22-mm cell culture filter.

**Cell culture.** RPTCs (NRK-52E, American Type Culture Collection, Rockville, MD) were cultured in DMEM/F12 medium containing 1.2 g/l sodium bicarbonate, 1 μg/ml endothelial growth factor, 5 μg/ml transferrin, 4 μg/ml dexamethasone, antibiotics, and 5% FBS. RPTCs were exposed to different concentrations of urinary proteins with or without TUPS for different times, then infected with recombinant adenoviral vectors at the indicated multiplicity and incubated for 48 h before experiments.

**Western blot analysis.** RPTCs and renal cortex tissues from patients or animals were lysed, and cellular proteins were extracted and underwent Western blot analysis with the primary anti-sEH (1:1,000), anti-β-actin (1:1,000), anti-α-SMA (1:2,000), and anti-GAPDH (1:1,000) and quantification by use of Scion Image (Scion, Frederick, MD).

**Real-time PCR.** Total RNA was isolated from cells with TRIzol reagent (Invitrogen). The isolated RNA was converted into cDNA and underwent quantitative RT-PCR by the Brilliant SYBR green QPCR system with GAPDH as an internal control. The primer sequences were for E-cadherin, 5'-TCGGTGCCGTATTGC-3' and 5'-GAATGCCCTCGTGTTG-3'; TGF-β1, 5'-AGGCGGTGTGCTGCTTGTG-3' and 5'-TGTTGCGGTCCACCCATACCGAC-3'; collagen I, 5'-CTCGGCTGTAGTCCACCCACGG-3' and 5'-ATGATGCGCAGTGGGAGC-3'; fibronectin, 5'-TGACTCTGGTCTTCCACCATAC-3' and 5'-TCGTTGCTCCTGTTCCGTCAGTGGT-3'; monocyte chemoattractant protein 1 (MCP-1), 5'-CAGCGAATGCTAGTACGC-3' and 5'-ATGCTGCACCTATCCACCAC-3'.

**Animal experiments.** All animal experimental protocols were approved by the Peking University Institutional Animal Care and Use Committee. Male BALB/c mice were kept in a 12-h light:12-h dark cycle and had free access to standard tap water for a 2-day adaptation. We divided 48 male mice into 2 groups for treatment (n = 24 each): ADR, tail-vein injection of 10 mg/kg ADR once; control, equivalent volume of saline. Each half of each group (n = 12) received by oral gavage the sEH inhibitor TUPS, 1 mg·kg⁻¹·day⁻¹ for 2 or 6 wk. Mice were housed in metabolic cages at weekly intervals to collect 24-h urine. After 2 or 6 wk of ADR administration, mice were killed, blood was collected, and kidneys were harvested. Histological examination was assessed by Masson, pulmonary artery smooth muscle, and α-SMA immunohistochemical staining on 2-μm-thick paraffin sections of kidney. Serum albumin and creatinine levels were measured by use of the commercial kits (albumin kit from BioSino Beijing, China; creatinine kit from Yantai, Ausbio Laboratories, Beijing, China) according to the manufacturers' instructions. Urinary protein concentration was expressed as protein (mg)/creatinine (mg).

**Statistical analysis.** Categorical variables were analyzed by χ² tests and continuous variables by one-way ANOVA (homogeneity of variance) or nonparametric t-test (heterogeneity of variance). Spear-
man rank correlation was used for correlation analysis. Statistical analysis involved use of SPSS 10.0 (SPSS, Chicago, IL). Data are expressed as means ± SD. A two-sided \( P < 0.05 \) was considered statistically significant.

RESULTS

**sEH expression and clinical characteristics of patients with glomerulonephritis.** To detect the expression of sEH in kidneys of patients with renal diseases, we collected renal biopsy specimens from 153 patients with glomerulonephritis. Among them, the pathological diagnoses were MCD (\( n = 28 \)), MN (\( n = 50 \)), and IgAN (\( n = 75 \)). Normal renal tissue specimens apart from tumor tissue of patients with renal carcinoma (\( n = 10 \)) served as controls. In both controls and renal disease tissue, sEH protein expression was found mainly in proximal tubules but not in glomeruli or in THP-stained distal tubules (Fig. 1A). According to the percentage and degree of positive-stained tubules, the immunohistochemistry results of sEH expression were classified from + to +++ (Fig. 1B). Tubular sEH level was higher in tissues from patients than in those from control subjects (2.82 ± 0.85 fold, \( P < 0.01 \)). As shown in Table 1, the levels of sEH in cases and control agreed with level of 24-h proteinuria, serum albumin level, incidence of edema, and renal function, but not incidence of hypertension, age, or sex.

The correlation analysis revealed that the extent of renal sEH expression in diseased tissue was positively correlated with 24-h proteinuria (\( r = 0.571, P = 0.001 \)) and incidence of edema (\( r = 0.446, P = 0.001 \)) and negatively with levels of serum albumin (\( r = -0.514, P = 0.001 \)) and serum creatinine but not incidence of renal failure (\( r = -0.265, P = 0.063 \)) or hypertension (\( r = -0.201, P = 0.124 \)).

**sEH expression and clinical characteristics in different glomerulonephritis.** The expression of sEH was significantly increased in patients with all three types of glomerulonephritis: MCD (3.12 ± 0.78), MN (3.02 ± 0.82), and IgAN (2.65 ± 0.89) (Fig. 1C). Similarly, Western blot analysis revealed a similar pattern of increased sEH protein expression in patients with different glomerulonephritides, with the expression of sEH greater in MCD and MN than in IgAN (Fig. 1D).
We previously reported that TUPS could significantly attenuate AngII-induced cardiac hypertrophy in vitro and in vivo (2). To decipher whether α-SMA upregulation and EMT of RPTECs was due to sEH upregulation, cells were administrated a selective sEH inhibitor TUPS, before treatment with urine proteins from different patients. Cell survivability was not affected by either urine proteins from patients or TUPS, as measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays (data not shown). Western blot analysis revealed that TUPS did not affect the increased expression of sEH by urine proteins (Fig. 3A); inhibition of sEH markedly reduced proteinuria-induced α-SMA upregulation. This result was further supported by confocal microscopy of immunostaining with anti-α-SMA antibody in proximal tubular epithelial cells (RPTECs). A: protein from patients’ urine was extracted and analyzed by 10% SDS-PAGE and Coomassie blue staining. Bovine serum albumin (BSA) was a control. RPTECs were treated with 10 mg/ml proteins from patients with MCD for different times (B) or with different concentrations of urine proteins from patients with MCD for 24 h (C). Western blot analysis of sEH, α-smooth muscle actin (α-SMA), and GAPDH proteins. Expression was normalized to that of GAPDH. Results are representative or means ± SD from at least 3 independent experiments (*P < 0.05 vs. 0 h).

Table 1. Clinical characteristics of patients with different tubular sEH level

<table>
<thead>
<tr>
<th>Clinical Information</th>
<th>sEH Expression in Renal Cortex of All Patients</th>
<th>+</th>
<th>++</th>
<th>+++</th>
<th>++++</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, male/female</td>
<td>6/5</td>
<td>20/21</td>
<td>37/30</td>
<td>17/17</td>
<td></td>
</tr>
<tr>
<td>Age, yr</td>
<td>31.3 ± 12.1</td>
<td>40.1 ± 13.4</td>
<td>36.2 ± 16.3</td>
<td>39.8 ± 15.5</td>
<td></td>
</tr>
<tr>
<td>24-h proteinuria, g/d*</td>
<td>2.7 ± 3.0</td>
<td>3.3 ± 2.8</td>
<td>4.8 ± 4.6</td>
<td>5.9 ± 4.0</td>
<td></td>
</tr>
<tr>
<td>Serum albumin level, U/l*</td>
<td>38.6 ± 8.8</td>
<td>33.5 ± 8.2</td>
<td>31.1 ± 8.9</td>
<td>27.5 ± 7.5</td>
<td></td>
</tr>
<tr>
<td>Incidence of edema*</td>
<td>45.5%</td>
<td>53.7%</td>
<td>62.7%</td>
<td>82.4%</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>27.3%</td>
<td>41.5%</td>
<td>32.8%</td>
<td>32.4%</td>
<td></td>
</tr>
<tr>
<td>Number of high Cr/normal*</td>
<td>5/6</td>
<td>16/25</td>
<td>18/49</td>
<td>6/28</td>
<td></td>
</tr>
<tr>
<td>Incidence of renal failure*</td>
<td>45.5%</td>
<td>39.0%</td>
<td>26.9%</td>
<td>17.6%</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD or number or percentage. sEH, soluble epoxide hydrolase. *P < 0.01, among different level of tubular sEH.

All patients with MCD, MN, or IgAN showed increased proteinuria, low serum albumin level, and edema (Table 2). However, proteinuria and edema was more severe and serum albumin level, hypertension, and renal failure were lower in patients with MCD and MN than IgAN (P < 0.05). Therefore, the expression of sEH in proximal tubules was related to the loss of protein in urine.

Proteinuria increased the expression of sEH in tubular epithelial cells in vitro. To investigate the role of proteinuria in sEH expression in renal diseases, we extracted proteins from urinary samples of patients with MCD, MN, and IgAN. Protein electrophoresis results showed the primary bands being albumin, of ~62 kDa, especially from MCD patients (Fig. 2A). Exposure of urinary proteins from patients with MCD to cultured RPTECs could time and dose dependently upregulate sEH (Fig. 2, B and C). Urine proteins from patients with MN or IgAN had a similar effect (data not shown). Interestingly, the expression of α-SMA, a marker of the process of epithelial-mesenchymal transition (EMT), was simultaneously upregulated with sEH in RPTECs with urine proteins from patients with MCD. The level of sEH began to increase at 12 h, and the expression of α-SMA increased significantly at 24 h, which indicates sEH was upregulated earlier than α-SMA (Fig. 2C).

sEH inhibition attenuated EMT and inflammatory reaction in RPTECs. We previously reported that TUPS could significantly attenuate AngII-induced cardiac hypertrophy in vitro and in vivo (2). To decipher whether α-SMA upregulation and EMT of RPTECs was due to sEH upregulation, cells were administrated a selective sEH inhibitor TUPS, before treatment with urine proteins from different patients. Cell survivability was not affected by either urine proteins from patients or TUPS, as measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays (data not shown). Western blot analysis revealed that TUPS did not affect the increased expression of sEH by urine proteins (Fig. 3A); inhibition of sEH markedly reduced proteinuria-induced α-SMA upregulation. This result was further supported by confocal microscopy of immunostaining with anti-α-SMA antibody in proximal tubular epithelial cells (RPTECs).

Table 2. sEH expression and clinical parameters in normal and diseased patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Normal</th>
<th>Total</th>
<th>MCD</th>
<th>MN</th>
<th>IgAN</th>
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<tbody>
<tr>
<td>sEH expression in cortex</td>
<td>+</td>
<td>10</td>
<td>11</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>++++</td>
<td>0</td>
<td>34</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>sEH expression</td>
<td>1.00 (n = 10)</td>
<td>2.8 ± 0.9* (n = 153)</td>
<td>3.1 ± 0.8** (n = 28)</td>
<td>3.0 ± 0.8** (n = 50)</td>
<td>2.6 ± 0.9* (n = 75)</td>
</tr>
<tr>
<td>24-h proteinuria, g/d</td>
<td>&lt;0.15</td>
<td>4.0 ± 0.3</td>
<td>6.9 ± 3.8†</td>
<td>5.5 ± 4.0†</td>
<td>3.9 ± 3.9</td>
</tr>
<tr>
<td>Serum albumin level, U/l</td>
<td>46 ± 6.4</td>
<td>31.3 ± 8.1</td>
<td>22.8 ± 4.9†</td>
<td>27.9 ± 5.7†</td>
<td>35.6 ± 9.1</td>
</tr>
<tr>
<td>Incidence of edema, %</td>
<td>0.00</td>
<td>59.2</td>
<td>96.0†</td>
<td>87.4†</td>
<td>33.8</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD. MCD, minimal change nephropathy; MN, membranous nephropathy; IgAN, IgA nephropathy. *P < 0.01 vs. normal group. †P < 0.05 vs. IgAN.
RPTECs (Fig. 3B). Furthermore, real-time PCR demonstrated that the urinary proteins from patients increased the mRNA level of MCP-1 and decreased that of E-cadherin, which was reversed by pretreatment with TUPS (Fig. 3C). The mRNA level of TGF-β1 in RPTECs was not altered by either treatment.

We studied next whether sEH was sufficient to induce EMT and an inflammatory effect by adenoviral overexpression of sEH in cultured RPTECs. The levels of protein of sEH were higher in Ad-sEH-infected cells than untreated or adenovirus encoding green fluorescent protein-infected controls (Fig. 4A). Noticeably, the expression of α-SMA and MCP-1 was increased in cells overexpressing sEH (Fig. 4, A–C). In contrast, the mRNA level of E-cadherin was decreased in Ad-sEH-infected RPTECs, which indicates the reduction of the cell junction (Fig. 4C) and suggests that sEH functions as a mediator of the proinflammatory effect of proteinuria in humans and is sufficient for inducing EMT in epithelial cells. Additionally, the mRNA levels of fibrosis markers, including collagen I, collagen III, and fibronectin, were also increased in Ad-sEH-infected RPTECs (Fig. 4D).

**sEH inhibition improved parameters in ADR-induced nephropathy in mice.** To explore whether sEH affects the development of proteinuria-mediated renal damage in vivo, we used the ADR-induced nephropathy mouse model. The ratio of protein to creatinine in urine and levels of serum albumin in controls, and a moderate improvement in ADR group but did not reach significance (Fig. 5A). Serum creatinine levels in diseased mice increased at 6 wk in the ADR group. Administration of TUPS significantly decreased the levels of serum creatinine at 6 wk after ADR injection (Fig. 5A, right).

Kidney cross sections were prepared for light microscopy. Sections were stained with Masson to evaluate the degree of renal histological injury and fibrosis. Interstitial fibrosis, inflammatory cell infiltration, and α-SMA staining of tubular cells were greater in kidney sections with ADR treatment, but significantly attenuated by TUPS (Fig. 5, B and C). For fibrosis markers, we found that the mRNA levels of collagen I and fibronectin, but not collagen III, were higher in the ADR group and reduced by TUPS treatment (Fig. 5D). Thus sEH inhibition significantly ameliorated inflammation, EMT, and fibrosis in ADR-induced nephropathy in mice.

Western blot analysis of the renal cortex revealed both sEH and α-SMA were significantly upregulated at 2 and 6 wk after ADR injection; TUPS had no effect on the expression of α-SMA for a short time (2 wk), but, consistent with the improvement in renal function and histology, TUPS could reduce α-SMA expression after long-term treatment (6 wk) (Fig. 6).

**DISCUSSION**

To explore the role of sEH in glomerulonephritis in humans, we found that 1) sEH expression was mainly located in proximal tubules and upregulated in patients; Western blot
analysis confirmed the elevated levels of sEH in patients with MN, MCD, and IgAN; 2) the level of sEH was positively correlated with severity of proteinuria and incidence of edema and negatively with serum albumin level; 3) the protein level of sEH was elevated along with increased \( \alpha \)-SMA protein and MCP-1 mRNA and decreased E-cadherin in RPTECs in vitro, whose effects were attenuated by an sEH inhibitor and mimicked with adenovirus-mediated sEH overexpression; and 4) in ADR-induced nephropathy mice, the sEH inhibitor did not improve proteinuria or serum albumin level but reduced long-term elevated serum creatinine level, interstitial inflammation, fibrosis, and \( \alpha \)-SMA expression. Thus upregulation of sEH in proximal tubular cells in chronic proteinuric kidney diseases may mediate the proteinuria-induced renal damage.

A comprehensive knowledge of tissue- or cell type-specific patterns of expression of sEH is essential for evaluating its functional significance, especially with the growing interest in its role in many biological activities. The enzyme activity of sEH in human tissue homogenates showed the kidney with the highest levels of enzyme activity (27). Yu and colleagues (37) reported that the expression of sEH localized largely in the renal vasculature, with relatively low levels in the surrounding tubules and glomeruli in human kidneys. The expression of sEH in renal arteries was localized mostly in smooth muscle layers of the arterial wall. The authors did not observe differences in sEH expression between normal and diseased human kidney tissue in the 15 samples examined (37). Using immunohistochemistry, Enayetallah et al. (7, 8) demonstrated abun-

Fig. 4. Role of sEH overexpression in cultured RPTECs. Confluent RPTECs were infected with recombinant adenovirus encoding sEH (Ad-sEH) or adenovirus encoding green fluorescent protein (Ad-GFP) for 48 h. A: Western blot analysis of sEH, \( \alpha \)-SMA and GAPDH proteins. B: confocal microscopy of \( \alpha \)-SMA in monolayer cells. Cell nuclei were stained by Hoechst (400×). C: real-time RT-PCR quantification of mRNA levels of E-cadherin, MCP-1, and TGF-\( \beta \). D: real-time RT-PCR quantification of mRNA levels of collagen I, collagen III, and fibronectin. Data are means ± SD of the relative mRNA normalized to that of GAPDH from at least 3 independent experiments (* \( P < 0.05 \)).

Fig. 5. sEH inhibition improved parameters in adriamycin (ADR)-induced nephropathy in mice. The ADR-induced nephropathy mouse model was established with tail vein injection of 10 mg/kg of ADR. The animals were divided into 4 groups (at least 6 mice in each group): control (Ctrl), ADR, TUPS, and ADR + TUPS. PBS treatment was a control. For TUPS treatment, sEH inhibitor TUPS (1.0 mg·kg\(^{-1}\)·day\(^{-1}\)) was given by oral gavage for 2 or 6 wk. A: 24-h urine protein level was determined every week; serum albumin and creatinine levels were measured 2 or 6 wk after ADR administration. B: Cross sections of mouse kidneys at 6 wk after ADR injection histochemically stained with Masson (fibrosis), pulmonary artery smooth muscle (inflammation), and \( \alpha \)-SMA (\( \times 400 \)). C: area of fibrosis, number of inflammatory cells and number of \( \alpha \)-SMA positive tubules in the cross-sections were measured. D: real-time RT-PCR quantification of mRNA levels of collagen I, collagen III, and fibronectin. Data are means ± SD of the relative mRNA normalized to that of GAPDH from at least 6 mice in each group (* \( P < 0.05 \); ** \( P < 0.01 \)).
DNA demethylation (1, 2, 38). Renal AngII, the intrarenal expression was upregulated by AngII and homocysteine in induced hypertension (17). We previously reported that sEH to toxic (28, 39), and sEH was the main effector in AngII-damage, diabetic nephropathy, and Cisplatin-induced nephropathy. The possible mechanism would be involved in elevated the intrarenal renin-angiotensin system and inflammation.

The elevated level of sEH can increase the hydrolysis of EETs to corresponding diols. EETs not only are involved in regulating vascular tone and antagonizing the vasoconstrictor actions of AngII but also directly influence tubular transport of sodium (13). In our study, the level of sEH was elevated along with increased EMT and inflammation in culture RPTECs, whose effects were mimicked with adenovirus-mediated sEH overexpression and attenuated by an sEH inhibitor in vitro and in ADR-induced nephropathy mice. Thus upregulation of sEH in chronic kidney diseases may mediate the proteinuria-induced renal damage via the decrease in the protective effect of EETs.

sEH inhibitors have been extensively studied in animal models of hypertension, with generally beneficial outcome. Interestingly, we found no correlation between sEH expression in kidney and the incidence of hypertension. Compared with patients with MCD and MN, those with IgAN had more hypertension but less sEH expression. One possible explanation is the different mechanism of hypertension and proteinuria. Many animal models used with sEH study were based on the hypertensive models, and albuminuria was the consequence of hypertension. Because hypertensive renal damage is not the index to perform a kidney biopsy, we did not collect samples with this condition. However, in patients with glomerulonephritis, proteinuria attributed to glomerular hypertension would be the response of chronic injury and progression of glomerulonephritis. However, hypertensive renal damage mainly affects vasculature; the elevated sEH level in tubular cells reflects the effects of infiltrated proteins in proteinuric disease, which caused renal EMT and interstitial inflammation.

Given that proximal tubules are more a “sufferer” than an “initiator” of proteinuria, the proteins passing through renal tubules may stimulate and damage tubular epithelial cells. We investigated the effects of urinary protein on the expression of sEH in cultured RPTECs in vitro and found the expression of sEH in this epithelial cell upregulated by urine proteins from patients, along with the upregulation of α-SMA and MCP-1 and down-regulation of E-cadherin, which could be largely attenuated by the sEH inhibitor. These results suggested that patients’ proteinuria-induced tubular EMT, inflammatory reaction were mediated, at least in part, by upregulation of sEH. Indeed, sEH overexpression could mimic the effect of the urine proteins and deteriorate the expression of those markers of EMT and inflammation, which suggests that elevated levels of sEH expression in renal proximal tubular epithelial cells is not necessary but is sufficient to trigger or aggregate the process of EMT and renal injury.

The ADR nephropathy is a common used model of chronic proteinuric renal disease. Owing to the cytotoxic effects of ADR in podocytes, mice develop significant proteinuria and a subsequent tubule-interstitial injury that mimics many features of chronic proteinuric renal disease in humans. Thus this model was reported to be useful in unraveling the pathogenesis of

Fig. 6. Expression of sEH and α-SMA in renal cortex of ADR-induced mouse nephropathy. Proteins were extracted from the renal cortex of animals in Fig. 5. Western blot analysis of sEH, α-SMA, and GAPDH proteins. A: representative results from each group of animals and the mean ± SD of the ratio of sEH to GAPDH (B) or α-SMA to GAPDH (C) from mice 2 and 6 wk (W2 and W6, respectively) after injection (**P < 0.01).
chronic proteinuric renal disease (36). In our clinical data, we found the elevated levels of sEH directly associated with the proteinuria in patients. Therefore, we used this model to further test our hypothesis that sEH upregulation in proximal tubules mediated epithelial cell EMT and injury. The expression of sEH increased 2 wk after ADR injection, with the increased proteinuria and decreased serum albumin. Inhibition of sEH activity did not affect severity of proteinuria or sEH expression, which suggests that sEH inhibition with TUPS did not cause increased protein infiltration in the acute phase. However, TUPS improved the renal function at 6 wk, as evidenced by decreased serum creatinine, interstitial fibrosis, inflammatory cell infiltration, and tubular α-SMA expression. Recently, inhibition of sEH failed to improve albuminuria in mice with 5/6 nephrectomy (18). Thus sEH inhibition protects renal function by improving tubular EMT, interstitial inflammation, and fibrosis induced by proteinuria but not proteinuria. Increasing protein infiltration may have direct toxic effects on tubular epithelial cells or on promoting renal interstitial inflammation via proinflammatory factors and profibrotic mediators and stimulating EMT. Tubulointerstitial damage and progressive fibrosis are common but relatively terminal pathways that lead to renal failure (10, 33).

In summary, sEH may participate in proteinuria-induced renal damage by triggering or aggravating the EMT of tubular cells and interstitial inflammatory and fibrosis process. sEH could be a potential therapeutic target for preventing the progression of chronic proteinuric kidney diseases.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


