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Enteropeptidase inhibitor SCO-792 effectively prevents kidney function decline and fibrosis in a rat model of chronic kidney disease

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ABSTRACT

Background. Inhibiting enteropeptidase, a gut serine protease regulating protein digestion, suppresses food intake and ameliorates obesity and diabetes in mice. However, the effects of enteropeptidase inhibition on kidney parameters are largely unknown. Here, we evaluated the chronic effects of an enteropeptidase inhibitor, SCO-792, on kidney function, albuminuria and kidney pathology in spontaneously hypercholesterolaemic (SHC) rats, a rat chronic kidney disease (CKD) model.

Methods. SCO-792, an orally available enteropeptidase inhibitor, was administered [0.03% and 0.06% (w/w) in the diet] to 20-week-old SHC rats showing albuminuria and progressive decline in glomerular filtration rate (GFR) for five weeks. The effects of SCO-792 and the contribution of amino acids to these effects were evaluated.

Results. SCO-792 increased the faecal protein content, indicating that SCO-792 inhibited enteropeptidase in SHC rats. Chronic treatment with SCO-792 prevented GFR decline and suppressed albuminuria. Moreover, SCO-792 improved glomerulosclerosis and kidney fibrosis. Pair feeding with SCO-792 (0.06%) was less effective in preventing GFR decline, albuminuria and renal histological damage than SCO-792 treatment, indicating the enteropeptidase-inhibition-dependent therapeutic effects of SCO-792. SCO-792 did not affect the renal plasma flow, suggesting that its effect on GFR was mediated by an improvement in filtration fraction. Moreover, SCO-792 increased hydrogen sulphide production capacity, which has a role in tissue protection. Finally, methionine and cysteine supplementation to the diet abrogated SCO-792-induced therapeutic effects on albuminuria.

Conclusions. SCO-792-mediated inhibition of enteropeptidase potently prevented GFR decline, albuminuria and kidney fibrosis; hence, it may have therapeutic potential against CKD.

Keywords: albuminuria, chronic kidney disease, enteropeptidase, fibrosis, glomerular filtration rate

INTRODUCTION

Chronic kidney disease (CKD) is characterized by a gradual loss of kidney functions, leading to end-stage renal disease (ESRD) that requires haemodialysis, peritoneal dialysis or renal transplantation [1]. Furthermore, CKD is a well-known risk factor for cardiovascular diseases [2]. Hence, preventing CKD progression will help maintain renal functions and improve the quality of life. Although inhibitors of the renin–angiotensin system and sodium–glucose co-transporter 2 show significant renoprotective effects [3, 4], many patients with CKD progress to ESRD. Thus, there is a need for therapeutic agents for CKD.

Glomerular hyperfiltration, which occurs in various clinical conditions such as kidney disease, hypertension and diabetes [5], contributes to glomerular injury including glomerulosclerosis in the remaining functional nephrons of patients with CKD [5–7]. It is induced by a high-protein diet or amino acid infusion, in both animal models and human subjects [8, 9]. In contrast to a high-fat or high-carbohydrate diet, a high-protein diet increases the glomerular filtration rate (GFR) by modulating renal haemodynamics and elevating intraglomerular pressure for efficient excretion of protein-derived nitrogenous waste products [10]. Along with an increase in albuminuria, high-protein diet-associated glomerular hyperfiltration may have deleterious, long-term consequences on various organs [10].

Enteropeptidase is a transmembrane serine protease that is localized to the brush border of the duodenal and jejunal mucosae, which are involved in the digestion of proteins in mammals [11–13]. It converts inactive trypsinogen into its active form, trypsin, resulting in the activation of digestive enzyme precursors produced in the pancreas (e.g. chymotrypsinogen, proelastase and procarboxypeptidases A and B). These activated enzymes facilitate protein breakdown, resulting in amino acid absorption in the gut [11–13]. Thus, enteropeptidase is an important upstream enzyme for protein digestion.

As amino acids are known to cause hyperfiltration, which is a risk factor for CKD, investigating the effects of pharmacological interventions to decrease amino acid intake is of interest. Hence, here, we aimed to evaluate the therapeutic effects of chronic enteropeptidase inhibition on kidney parameters in a rat CKD model. SCO-792, an orally available enteropeptidase inhibitor, is under clinical evaluation for diabetes, obesity and diabetic kidney disease [14]. Therefore, we first evaluated SCO-792-mediated *in vivo* enteropeptidase inhibition before examining its effects on kidney functions, albuminuria and pathology. We then investigated the role of amino acids in SCO-792-induced effects in the rat CKD model.

KEY LEARNING POINTS

What is already known about this subject?

- increased protein intake and circulating amino acid levels are suggested to be deleterious to the kidney functions in chronic kidney disease (CKD);
- however, the therapeutic effects of pharmacological interventions on these events are largely unknown.
- we evaluated the effects of inhibiting gut enteropeptidase, the most upstream enzyme regulating protein digestion, in CKD rats.

What this study adds?

- we administered SCO-792, an enteropeptidase inhibitor under clinical evaluation, to CKD rats;
- SCO-792 treatment prevented glomerular filtration rate decline, improved albuminuria, ameliorated glomerulosclerosis and alleviated kidney fibrosis in CKD rats; and
- these findings suggest that enteropeptidase inhibition is likely effective in improving kidney functions in CKD.

What impact this may have on practice or policy?

- inhibitors of the renin–angiotensin system and sodium–glucose co-transporter 2 show substantial renoprotective effects in patients with CKD;
- however, new treatment options are necessary owing to the fact that many patients with CKD progress to end-stage renal disease; and
- our study provides a new renoprotective strategy for patients with CKD.

MATERIALS AND METHODS

Reagents

All reagents were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) or Sigma-Aldrich (St Louis, MO, USA), unless otherwise indicated. SCO-792 was synthesized by Takeda Pharmaceutical Company Limited (Tokyo, Japan).

Animals

Spontaneously hypercholesterolaemic (SHC) male rats were obtained from RABICS, Ltd (Kanagawa, Japan) and normal male Sprague-Dawley (SD) rats from CLEA Japan, Inc. (Tokyo, Japan). SHC rats were established as a model of spontaneous hyperlipidaemia with albuminuria and histological changes mimicking focal segmental glomerulosclerosis on the genetic background of the SD rats [15]. All rats were housed in a room with controlled temperature (23°C), humidity (55%) and lighting (lights remained on between 7:00 a.m. and 7:00 p.m.). All rats had free access to a standard laboratory chow diet (CE-2, CLEA) and tap water. The care of rats and use of the experimental protocols were approved by the Institutional Animal Care and Use Committee in Shonan Health Innovation Park accredited by the American Association for Accreditation of Laboratory Animal Care. SHC rats show albuminuria and hyperlipidaemia by 8 weeks of age, and their renal functions start to decline around 16 weeks of age with the histological changes of glomerulosclerosis and tubular dilatation, which are major histological features of patients with CKD [16]. Thus, SHC rats are considered a good model for investigating the effects on kidney functions, severe albuminuria and pathology in CKD [17].

Experimental design

All rats were housed individually in standard animal cages and used in the study after 1 week of acclimation. Twenty-week-old male SHC rats, which showed a progressive decline in the kidney function, were allocated to four groups ($n = 10$) based on their GFR, urine albumin-to-creatinine ratio (UACR), plasma creatinine (pCre), blood urea nitrogen (BUN) and body weight. SD rats of the same age were used as normal control ($n = 6$).

Main study. The SHC rats in each group had free access to either diet alone (vehicle and pair-fed) or diet containing 0.03% (w/w) or 0.06% (w/w) SCO-792. Pair-fed SHC rats received the same amount of diet as the 0.06% SCO-792-treated rats but without the drug. Normal rats had free access to diet alone. We determined the doses of SCO-792 based on our previous study using a rat model with diabetic kidney disease [18]. The first treatment day was designated as Day 0. After treatment initiation, body weight and food intake changes were monitored twice a week for 4 weeks. On Days 14 and 28, GFR, pCre and BUN were measured. Renal plasma flow was measured on Day 28. Faeces were collected on Day 21 for 24 h to determine faecal protein content. To measure urinary albumin excretion, urine was collected on Day 30 for 24 h. On Day 36, the rats were anaesthetized with sodium pentobarbital (50 mg/kg i.p.) and sacrificed; then, peripheral blood was sampled and the left kidney was harvested, weighed and processed for the subsequent assays. Plasma branched-chain amino acids (BCAAs), triglyceride (TG) and total cholesterol (TC) were measured. The ratio of cortex and medulla in the renal tissues sampled reflected that of the entire kidney. The samples were immediately immersed in liquid nitrogen and stored at −80°C for the analysis of gene expression and measurement of kidney collagen content and hydrogen sulphide production capacity.

Additional experiment. This experiment was conducted to investigate the contribution of the reduction in methionine (Met) and cysteine (Cys) intake by SCO-792 to the therapeutic efficacy of SCO-792. Each group of SHC rats receiving the drug treatments had free access to CE-2 powder chow containing 0.06% SCO-792 or CE-2 powder chow containing 0.06% SCO-792 supplemented with Met/Cys. Met and Cys were used at the concentrations of 0.65% (w/w) and 0.58% (w/w) to supplement the diet of the SCO-792 + low-Met/Cys-treated group and at the concentrations of 1.95% (w/w) and 1.73% (w/w) to supplement the diet of the SCO-792 + high-Met/Cys-treated group, respectively. To measure UACR, urine was collected on Day 22. On Day 24, the rats were anaesthetized with pentobarbital and sacrificed; then, the left kidney was harvested and weighed. Some pieces of the kidney were immediately immersed in liquid nitrogen and stored at −80°C for the gene expression analysis described below.

Faecal protein content

The collected faeces samples were lyophilized and powdered; the powder samples were homogenized in 0.5 N NaOH solution. The homogenates were centrifuged, and the concentration of proteins in the supernatants was determined using the DC Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Faecal protein content was calculated as protein content per gram of faeces.

GFR and renal plasma flow

GFR and renal plasma flow were measured using inulin–fluorescein isothiocyanate (FITC) (Sigma-Aldrich) and 10% (w/v) sodium *para*-aminohippurate injection (PAH, Daiichi Sankyo Company, Ltd, Tokyo, Japan), respectively. Inulin–FITC was dissolved in saline to prepare a 5% (w/v) solution. To measure GFR, 5% inulin–FITC solution was injected into the tail vein at 0.8 mL/kg. At 20, 40, 60 and 80 min after injection, blood was collected from the tail vein to measure the inulin–FITC concentration. To measure GFR and renal plasma flow, 5% inulin–FITC solution and 10% PAH were mixed at a ratio of 8:3, and the mixed solution was injected into the tail vein at 1.1 mL/kg. At 5, 10, 15, 20, 40, 60 and 80 min after injection, blood was collected from the tail vein. GFR and renal plasma flow were calculated from inulin–FITC clearance and PAH clearance, respectively [19]. The filtration fraction was calculated by dividing the GFR by renal plasma flow.

Biochemical parameters

Plasma parameters (pCre, BUN, TG, TC and albumin) and urine parameters (albumin and creatinine) were measured using the Hitachi 7180 Chemistry Analyser (Hitachi, Ltd, Tokyo, Japan). Creatinine, BUN, TG and TC were measured using enzymatic methods [20–23] and albumin was measured using the immunonephelometry method. Plasma BCAAs were measured using an liquid chromatography with tandem mass spectrometry system (Xevo TQ-S micro, Waters, Milford, MA, USA).

Histological evaluation

Formalin-fixed, paraffin-embedded kidney tissues were sectioned to 4-μm thickness at the midhorizontal plane for light microscopic examination. The specimens were stained with haematoxylin and eosin (HE), Sirius red (SR) and Masson's trichrome (MT) stains. The stained sections were digitized using the NanoZoomer digital slide scanner (Hamamatsu Photonics K.K., Hamamatsu, Japan) with a 40× optical lens. Dilatation of tubules and tubular basophilia were assessed using the HE-stained specimens, interstitial fibrosis was assessed using the SR-stained specimens and glomerulosclerosis was assessed using the MT-stained specimens. They were semi-quantitatively graded from 0 to 4 (0, not remarkable; 1, minimal; 2, mild; 3, moderate; 4, marked). These specimens were evaluated using a light microscope by a pathologist and assessed under blind conditions.

Kidney collagen content

The kidney tissue samples were incubated in 6 N HCl at 95°C for 20 h to hydrolyse collagen to hydroxyproline. After centrifugation, hydroxyproline content in the supernatants was quantified using the Total Collagen Assay Kit (QuickZyme Biosciences, Leiden, The Netherlands). The kidney collagen

content was calculated as the total content of collagen in the left kidney.

Hydrogen sulphide production capacity

Hydrogen sulphide production capacity was measured using the lead sulphide method [24]. Each kidney tissue sample was homogenized in passive lysis buffer (Promega Corp., Madison, WI, USA). An equal volume of a mixture containing PBS, 1 mM pyridoxal 5′-phosphate and 10 mM Cys was added to the homogenate. Lead acetate indicator paper (Whatman, Sigma-Aldrich) was placed above the liquid phase and incubated for 5.5 h at 37°C until the paper darkened due to the formation of lead sulphide. The indicator papers were photographed using a digital camera, and the obtained images were analysed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) to measure the area and density of the dark-coloured areas of the indicator papers. Hydrogen sulphide production capacity was calculated as the capacity per tissue weight.

Statistical analysis

Statistical significance was first analysed using Bartlett's test for homogeneity of variances, followed by Williams' test ($P \geq 0.05$) or Shirley–Williams test ($P < 0.05$) for dose-dependent studies. Alternatively, statistical significance between two groups was analysed using the *F*-test for homogeneity of variances, followed by Student's *t*-test ($P \geq 0.2$) or Aspin–Welch test ($P < 0.2$). Williams' and Shirley–Williams tests were conducted using a one-tailed significance level of 2.5% (0.025). Other tests were conducted using a two-tailed significance level of 5% (0.05). All data are presented as mean ± standard deviation.

Methods for gene expression analysis are provided in the Supplementary Methods.

RESULTS

SCO-792 inhibited enteropeptidase

The average dose of SCO-792 for the rats in the SCO-792 (0.03%) and SCO-792 (0.06%) groups was 14.9 ± 1.2 and 26.0 ± 1.9 mg/kg/day, respectively. We found that SCO-792 increased faecal protein content, which indicated the inhibition of protein digestion in the gut (Figure 1A). We measured plasma BCAA concentrations as a representative of amino acids on Day 36. SCO-792 dose-dependently decreased plasma BCAA concentration (Figure 1B). SCO-792 dose-dependently decreased food intake and body weight compared with those of the vehicle-treated SHC rats (Figure 1C and D). In the pair-fed group, the faecal protein levels were unchanged, and plasma BCAA concentrations tended to decrease compared with those in the vehicle-treated SHC rats (Figure 1A and B). The plasma TG and TC levels increased in the SHC rats compared with those in the normal rats (Supplementary data, Figure S1A and B). SCO-792 did not affect the plasma TG level and slightly decreased plasma TC to the same level as pair-fed treatment (Supplementary data, Figure S1A and B). Throughout the study, there were no abnormal findings in the SCO-792-treated SHC rats.

SCO-792 improved the kidney function

Treatment with SCO-792 was started at 20 weeks of age. At the start, the GFR of the SHC rats was 6.17 ± 0.78 mL/min/kg, which was significantly lower than that of normal rats (7.34 ± 0.69 mL/min/kg). During the 4-week treatment period, although the GFR was maintained in normal rats, it declined with age in the SHC rats (Figure 2A). At 4 weeks, the SHC rats showed decreased GFR, renal plasma flow and filtration fraction compared with those in the normal rats (Figure 2A–C). SCO-792 dose-dependently prevented the decline in GFR and suppressed any increase in the pCre level (Figure 2A and D). At 4 weeks, the GFR of the vehicle-, SCO-792 (0.03 and 0.06%)- and pair-fed-treated SHC rats was 2.53 ± 1.06, 4.43 ± 1.25, 5.53 ± 0.59 and 3.72 ± 1.37 mL/min/kg, respectively (Figure 2A). Although SCO-792 did not change the renal plasma flow, it improved filtration fraction (Figure 2B and C). The SHC rats exhibited pronounced albuminuria compared with normal rats (Figure 2E). SCO-792 dose-dependently suppressed albuminuria in the SHC rats (Figure 2E). The plasma albumin level was not changed by SCO-792 treatment (Supplementary data, Figure S1C). Pair feeding with SCO-792 (0.06%) slightly improved the GFR, but the improvement with SCO-792 (0.06%) was more than that with pair feeding. Pair feeding did not alter the filtration fraction, pCre and albuminuria compared with vehicle treatment (Figure 2A and C–E).

SCO-792 ameliorated renal histological damage

At the end of the study, the kidney of rats was histologically evaluated. Glomerulosclerosis, dilatation of tubules, tubular basophilia and interstitial fibrosis, all of which are major histological features of CKD, were observed in the SHC rats (Figure 3A–G). Five-weeks of SCO-792 (0.06%) treatment improved glomerulosclerosis and tubular damage, including dilatation of tubules and tubular basophilia (Figure 3A, B and D–F). Moreover, SCO-792 dose-dependently decreased fibrosis (Figure 3C and G). It suppressed the mRNA expression of fibrosis-related genes such as *Col1a1*, *Tgfb1* and *Fn1*, and collagen content in the kidney compared with vehicle treatment (Figure 3H–K). Pair feeding with SCO-792 (0.06%) did not affect the scores for glomerulosclerosis, dilatation of tubules, tubular basophilia and fibrosis, but partially suppressed the mRNA levels of fibrosis-related genes and collagen content compared with vehicle treatment (Figure 3). SCO-792 potently reduced the fibrosis score and *Col1a1* mRNA level in comparison with those in the pair-fed rats (Figure 3G and H).

SCO-792 increased hydrogen sulphide production capacity

The restriction of sulphur-containing amino acids, such as Met and Cys, increases hydrogen sulphide production capacity, which induces a protective effect on tissues [24]. As enteropeptidase inhibition decreases amino acid uptake into the circulation, we measured the hydrogen sulphide production capacity in the kidney of the rats. Both mRNA level of *Cth*, which encodes a hydrogen sulphide-producing enzyme, and hydrogen sulphide production capacity in the kidney were decreased in the SHC rats compared with those in the normal rats

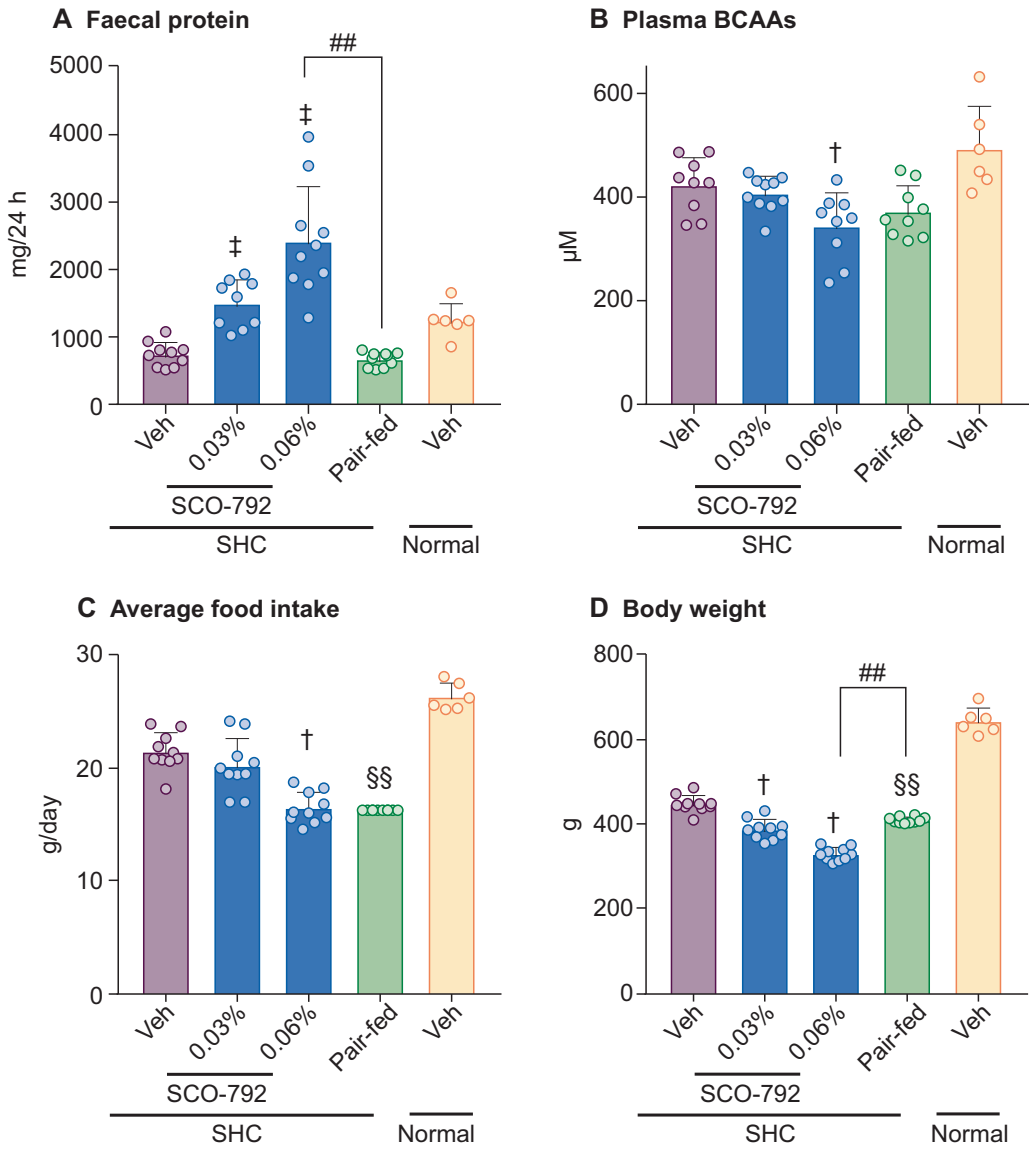

FIGURE 1: Effects of SCO-792 on faecal protein, plasma BCAAs, average food intake and body weight in SHC rats. SHC rats were treated with SCO-792 (0.03% or 0.06% added to the diet) for 5 weeks. (**A**) Faecal protein levels were measured on Day 21. (**B**) Plasma BCAAs were measured on Day 36. Food intake and body weight were monitored during the study; (**C**) average food intake was calculated; and (**D**) body weight was measured on Day 28. SHC, SHC rats; Normal, normal rats; Veh, vehicle; Pair-fed, the group fed the same amount of food as the SCO-792 (0.06%) group. The values are expressed as mean ± standard deviation ($n = 9$–10 for SHC rats and 6 for normal rats). $^{\dagger}P < 0.025$ versus vehicle-treated SHC rats using Williams' test. $^{\ddagger}P < 0.025$ versus vehicle-treated SHC rats using Shirley–Williams test. $^{\#\#}P < 0.01$ versus SCO-792 (0.06%) using Aspin–Welch test. $^{\S\S}P < 0.01$ versus vehicle-treated SHC rats using Aspin–Welch test.

(Figure 4). Interestingly, SCO-792 upregulated the mRNA levels of *Cth* and hydrogen sulphide production capacity in the kidney of SHC rats (Figure 4). Although the pair-fed rats with SCO-792 (0.06%) showed elevated mRNA levels of *Cth* and hydrogen sulphide production capacity, the effects were more evident in the SCO-792-treated rats (Figure 4).

Met and Cys supplementation alleviated albuminuria-suppressive effect of SCO-792

We evaluated the contribution of sulphur-containing amino acids on the therapeutic efficacy of SCO-792. Three weeks of Met/Cys supplementation in the diet suppressed the increase in the *Cth* mRNA level in the kidney by SCO-792 treatment (Figure 5A). Moreover, Met/Cys supplementation almost alleviated the reduction in UACR induced by SCO-792 treatment (Figure 5B).

DISCUSSION

Enteropeptidase is a key enzyme that regulates protein breakdown in the gut. Here, we evaluated the effect of SCO-792, an enteropeptidase inhibitor, in a rat model of CKD. This study is the first to reveal the therapeutic potential of enteropeptidase inhibition in CKD. SCO-792 inhibited GFR decline probably by improving the renal filtration fraction and suppressed albuminuria. Moreover, SCO-792 improved glomerulosclerosis and interstitial fibrosis in the kidney. Additionally, increased

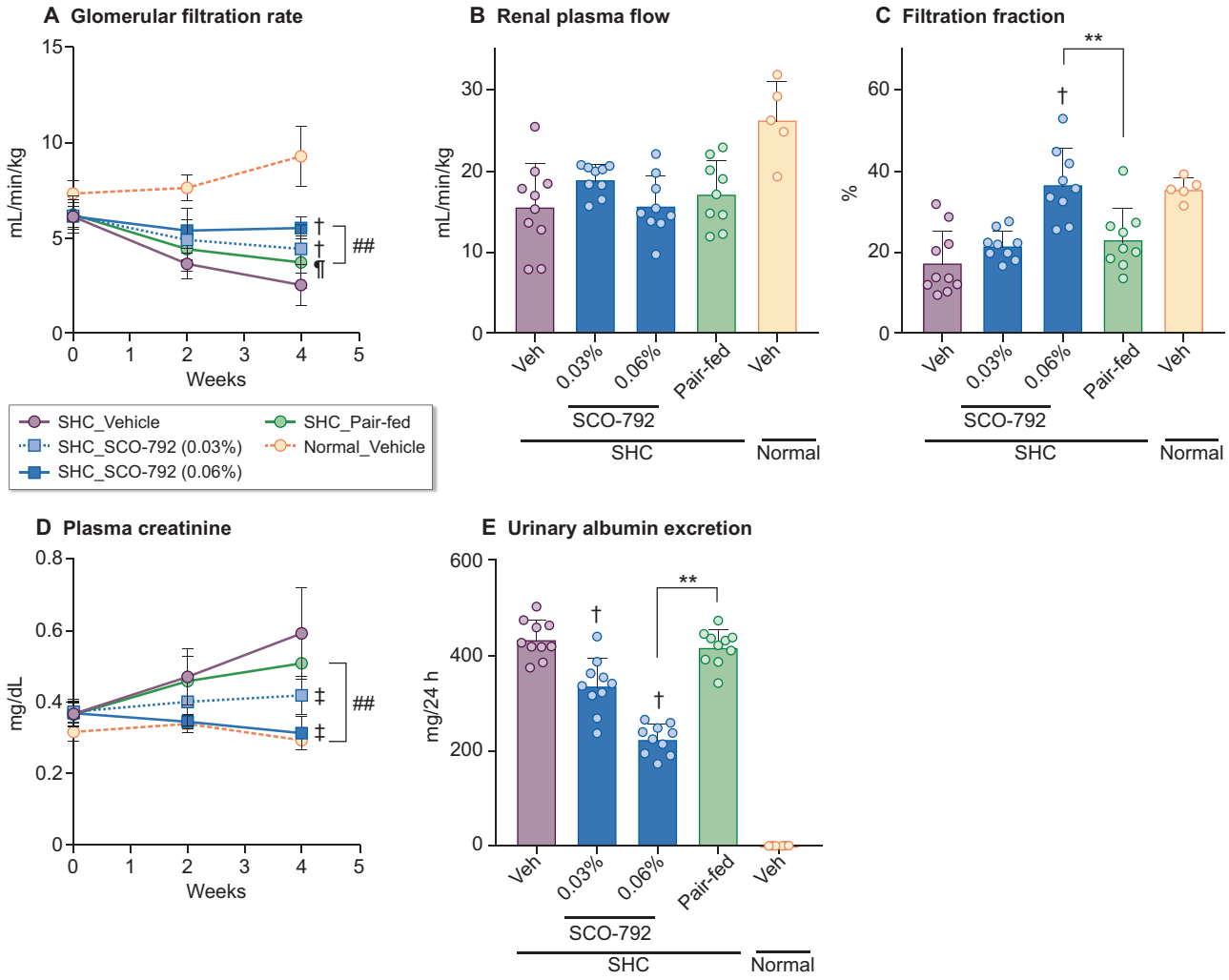

FIGURE 2: Effects of SCO-792 on the GFR, renal plasma flow, filtration fraction, pCre and urinary albumin excretion in SHC rats. SHC rats were treated with SCO-792 (0.03% or 0.06% added to the diet) for 5 weeks. (**A**) GFR was measured before the study and on Days 14 and 28. (**B**) Renal plasma flow and (**C**) filtration fraction were examined on Day 28. (**D**) pCre was measured before the study and on Days 14 and 28. (**E**) Urinary albumin excretion for 24 h was measured on Day 30. Filtration fraction was calculated by dividing GFR with renal plasma flow. SHC, SHC rats; Normal, normal rats; Veh, vehicle; Pair-fed, the group fed the same amount of food as the SCO-792 (0.06%) group. The values are expressed as mean ± standard deviation ($n = 7$–10 for SHC rats and 5–6 for normal rat). $^{\dagger}P < 0.025$ versus vehicle-treated SHC rats using Williams' test. $^{\ddagger}P < 0.025$ versus vehicle-treated SHC rats using Shirley–Williams test. $^{**}P < 0.01$ versus SCO-792 (0.06%) using Student's *t*-test. $^{\#\#}P < 0.01$ versus SCO-792 (0.06%) using Aspin–Welch test. $^{¶}P < 0.05$ versus vehicle-treated SHC rats using Student's *t*-test.

hydrogen sulphide production capacity induced by the reduction in the intake of Met and Cys may play a role in the therapeutic effect of SCO-792 on albuminuria.

The primary endpoint for patients with CKD in clinical trials is the composite outcome of time to doubling of the baseline serum creatinine level or the development of ESRD that requires renal replacement therapy due to GFR reduction [25]. Therefore, preclinical models that mimic clinical CKD pathology along with a decrease in the GFR over a short period are useful to evaluate anti-CKD drugs. Nevertheless, the development of a preclinical model that simulates the clinical CKD situation is difficult; this has hindered the discovery and development of anti-CKD drugs [26, 27]. Here, the SHC rats showed a decrease in the GFR accompanied by glomerulosclerosis and fibrosis with age. Hence, SHC rats are considered a useful preclinical model to evaluate the therapeutic efficacy of test drugs in CKD. Here, 20-week-old SHC rats showed lower GFR than that of normal rats before the study. SCO-792-mediated enteropeptidase inhibition potently prevented any GFR decline, whereas GFR declined progressively in the vehicle-treated SHC rats during the study. Moreover, interstitial fibrosis, which is the final common pathway to end-stage renal failure, was suppressed by SCO-792. These data suggest that SCO-792 may be a novel and promising treatment option for patients with CKD.

In a 5-week study of SHC rats, SCO-792 decreased food intake and exhibited potent renoprotective effects. Reports have suggested that restricting calorie intake may be beneficial for kidney protection [28, 29]. Hence, we employed a pair-fed group to examine the contribution of SCO-792-induced food intake reduction on renoprotective effects. SCO-792 potently suppressed GFR decline, albuminuria and kidney damage compared with those in the pair-fed group.

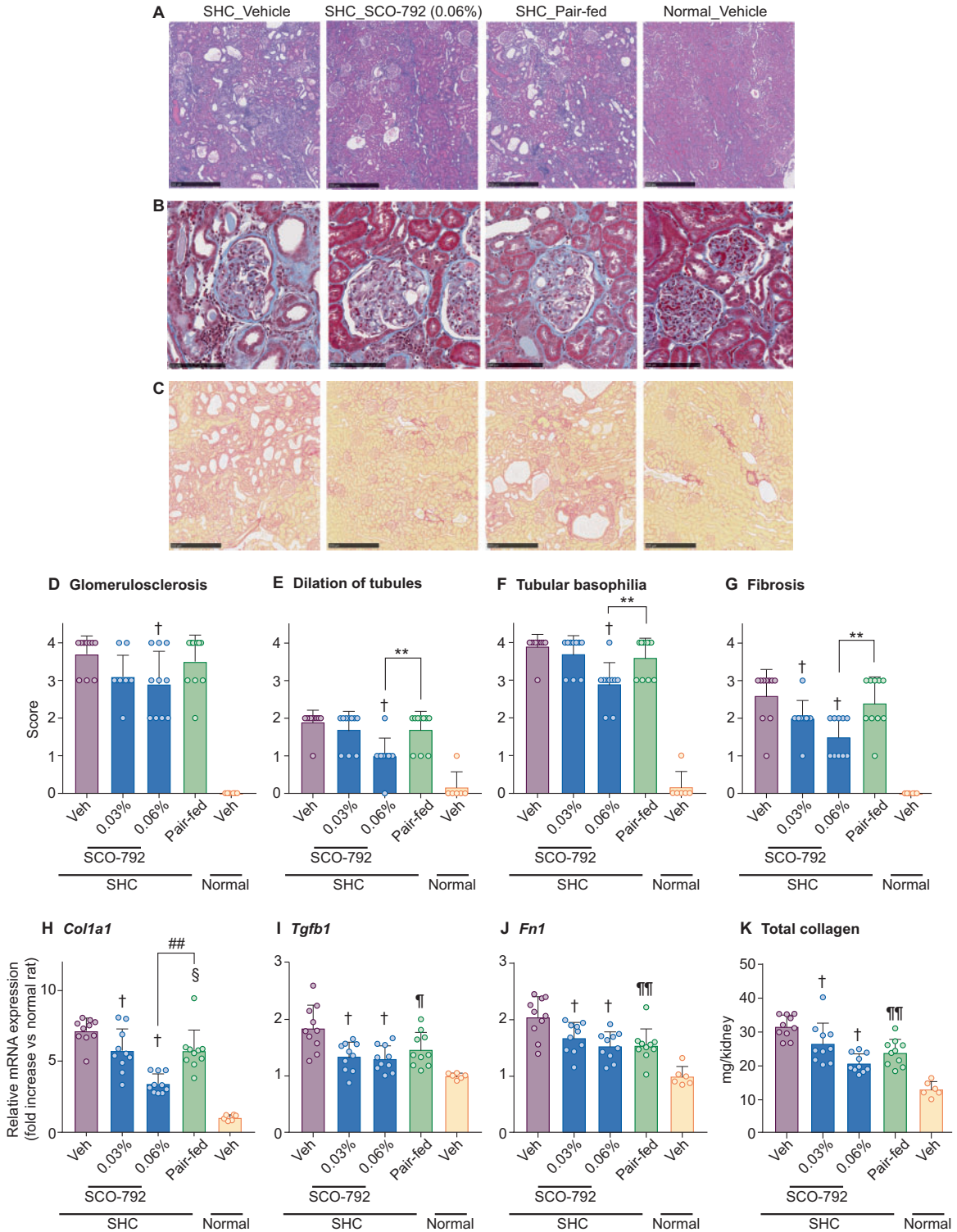

FIGURE 3: Effects of SCO-792 on renal histological damage in SHC rats. SHC rats were treated with SCO-792 (0.03% or 0.06% added to the diet) for 5 weeks. Representative microphotographs of the renal cortex stained with (**A**) HE, (**B**) MT or (**C**) SR stains are shown. (**D**) Glomerulosclerosis was assessed using the MT-stained specimens, (**E**) dilatation of tubules and (**F**) tubular basophilia was assessed using the HE-stained specimens and (**G**) interstitial fibrosis was assessed using the SR-stained specimens; they were semi-quantitatively graded from 0 to 4 (0, not remarkable; 1, minimal; 2, mild; 3, moderate; 4, marked). mRNA levels of (**H**) *Col1a1*, (**I**) *Tgfb1* and (**J**) *Fn1* and (**K**) content of total collagen in the kidney were examined. Scale bar = 500 μm for (A) and (C) and 100 μm for (B). SHC, SHC rats; Normal, normal rats; Veh, vehicle; Pair-fed, the group fed the same amount of food as the SCO-792 (0.06%) group. The values are expressed as mean ± standard deviation ($n = 10$ for SHC rat and 6 for normal rat). $^{\dagger}P < 0.025$ versus vehicle-treated SHC rats using Williams' test. $^{\ddagger}P < 0.025$ versus vehicle-treated SHC rats using Shirley–Williams test. $^{**}P < 0.01$ versus SCO-792 (0.06%) using Student's *t*-test. $^{\#\#}P < 0.01$ versus SCO-792 (0.06%) using Aspin–Welch test. $^{¶}P < 0.05$ and $^{¶¶}P < 0.01$ versus vehicle-treated SHC rats using Student's *t*-test. $^{§}P < 0.05$ versus vehicle-treated SHC rats using Aspin–Welch test.

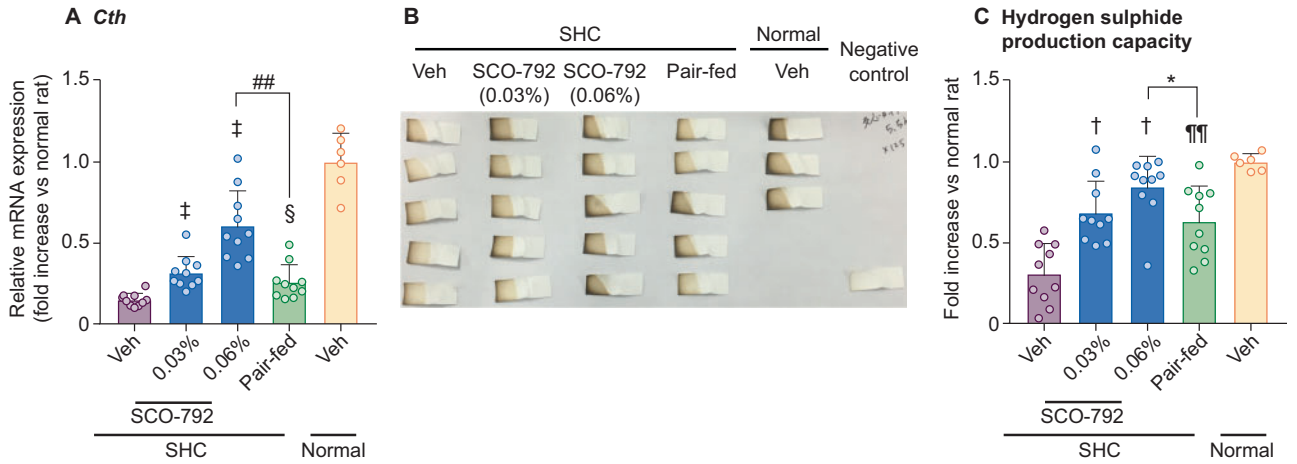

FIGURE 4: Effects of SCO-792 on the *Cth* mRNA levels and hydrogen sulphide production capacity in SHC rats. SHC rats were treated with SCO-792 (0.03% or 0.06% added to the diet) for 5 weeks. (**A**) Cystathionine gamma-lyase (*Cth*) mRNA level in the kidney was analysed. (**B**) Hydrogen sulphide production capacity in the kidney was detected as brown-black colouration of lead sulphide paper and (**C**) quantitatively analysed. SHC, SHC rats; Normal, normal rats; Veh, vehicle; Pair-fed, the group fed the same amount of food as the SCO-792 (0.06%) group. The values are expressed as mean ± standard deviation ($n = 10$ for SHC rat and 6 for normal rat). $^{\dagger}P < 0.025$ versus vehicle-treated SHC rats using Williams' test. $^{\ddagger}P < 0.025$ versus vehicle-treated SHC rats using Shirley–Williams test. $^{*}P < 0.05$ versus SCO-792 (0.06%) using Student's *t*-test. $^{\#\#}P < 0.01$ versus SCO-792 (0.06%) using Aspin–Welch test. $^{¶¶}P < 0.01$ versus vehicle-treated SHC rats using Student's *t*-test. $^{\S}P < 0.05$ versus vehicle-treated SHC rats using Aspin–Welch test.

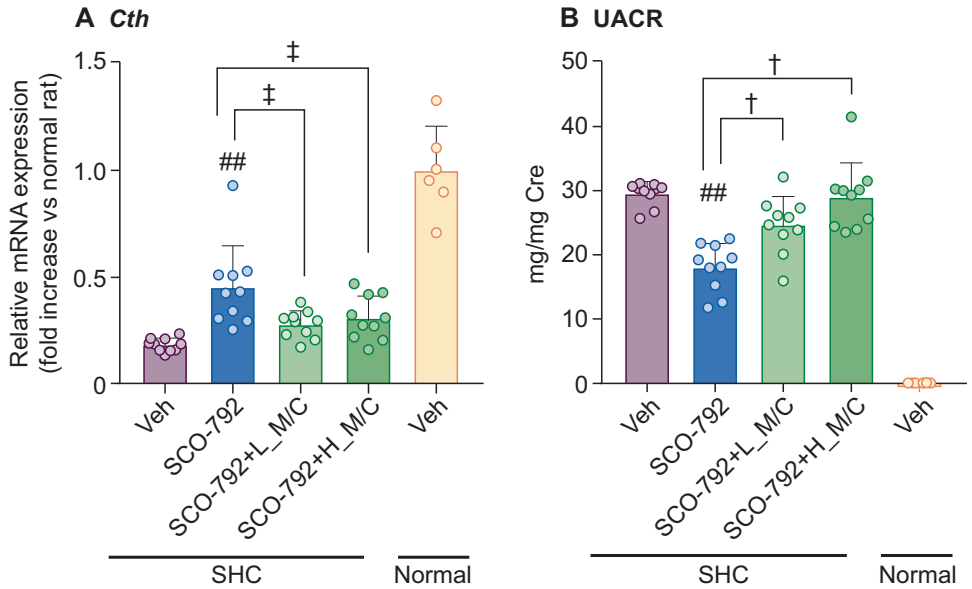

FIGURE 5: Effects of Met and Cys supplementation on SCO-792-induced kidney protection in SHC rats. SHC rats were treated with SCO-792 (0.06% added to the diet) or Met and Cys supplementation to SCO-792 (0.06%)-containing diet for 3 weeks. (**A**) Cystathionine gamma-lyase (*Cth*) mRNA level in the kidney and (**B**) UACR were examined. L_M/C: 0.65% Met and 0.58% Cys were added to the diet. H_M/C: 1.95% Met and 1.73% Cys were added to the diet. SHC, SHC rats; Normal, normal rats; Veh, vehicle; SCO-792, SCO-792 (0.06%). The values are expressed as mean ± standard deviation ($n = 10$ for SHC rat and 6 for normal rat). $^{\#\#}P < 0.01$ versus vehicle-treated SHC rats using Aspin–Welch test. $^{\dagger}P < 0.025$ versus SCO-792-treated group using Williams' test. $^{\ddagger}P < 0.025$ versus SCO-792-treated group using Shirley–Williams test.

Considering that pair feeding may be partially responsible for the observed therapeutic effects of SCO-792, it is likely that the therapeutic efficacy of SCO-792 may be associated with mechanisms that are both dependent and independent of food intake reduction.

Amino acids are known to cause hyperfiltration in both healthy subjects and patients with CKD [8, 30]. Consistently, studies using rodent models suggest the beneficial effects of a low-protein diet on renal functions [31, 32]. Moreover, a low-protein diet reduces the introduction of renal replacement therapy and mortality in patients with CKD [33]. Thus, SCO-792 might have protected renal functions by decreasing amino acid intake into the circulation in SHC rats in this study.

The restricted intake of specific amino acids, particularly Met, has been demonstrated to protect the kidney [34]. Additionally, the restricted intake of sulphur-containing amino

acids increases hydrogen sulphide production capacity, which has anti-inflammatory, antioxidant and antifibrotic effects on tissues [24, 35]. Here, SCO-792 administration for 5 weeks increased the mRNA levels of *Cth*, a hydrogen sulphide-producing enzyme, and elevated hydrogen sulphide production capacity in the kidney. Considering the role of enteropeptidase in protein digestion, SCO-792 may restrict the generation of sulphur-containing amino acids, and thereby increase *Cth* mRNA and hydrogen sulphide production capacity. Dietary supplementation of Met and Cys, which likely bypasses the effects of enteropeptidase inhibition, alleviated the reduction in albuminuria in the SCO-792-treated SHC rats. Thus, SCO-792-induced improvement in albuminuria may be a result of decreased intake of Met and Cys.

Although we demonstrated that SCO-792 treatment is highly effective in improving kidney parameters in a rat model of CKD, the precise biological mechanisms by which SCO-792 induces these therapeutic effects remain unclear. Thus, further studies are required to understand how enteropeptidase inhibition results in potent kidney protection effects.

This is the first study to show that enteropeptidase inhibition improved kidney function, albuminuria and renal pathology in a rat CKD model. Our findings suggest that SCO-792-mediated inhibition of enteropeptidase may have therapeutic potential in patients with CKD.

SUPPLEMENTARY DATA

Supplementary data are available at ndt online.

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AUTHORS' CONTRIBUTIONS

The research study was designed by all authors. Experiments were conducted by Y.K. Data were analysed and interpreted by all authors. The manuscript was written by Y.K. and Y.M., and important intellectual content of the manuscript was reviewed and revised by all authors. All authors have agreed to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

CONFLICT OF INTEREST STATEMENT

Y.K., J.S., T.S., Y.I., A.K., Y.M. and M.W. are/were employees of SCOHIA PHARMA, Inc.

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