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Original Paper

Riligustilide Attenuated Renal Injury by the Blockade of Renin

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Key Words
Riligustilide • Renin • Renal Injury • CREB • Nephropathy

Abstract
Background/Aims: Nephropathy related with renin can be alleviated with ACE-inhibitors or AT1R blockers, whereas they might be ineffective after long-term administration because of a feedback production of enhanced renin. Therefore, it is urgent to develop a new category of anti-nephropathy medicine directly targeting renin. Riligustilide (C20), originally isolated from the Chinese herb Ligusticumporteri, a rhizome, was confirmed effective against many diseases. ***Methods:*** The therapeutic effect of C20 on renal injury and its underlying mechanism were investigated in three different nephrotic models, which were spontaneously hypertension rats (SHR) model, diabetic nephropathy in BTBR ob/ob mice model and 5/6-nephrectomized (5/6NX) rats model. ***Results:*** The intensity of kidney fibrosis was extensively decreased in the C20-treated rats compared to the vehicle animals. C20 significantly alleviated renal injury much more in 5/6 NX rats than in vehicle group. The rats in 5/6 NX without administrated C20 developed albuminuria earlier with more severe symptoms. Additionally, our findings showed that C20 down-regulated the renin expression and relocation of CREB-CBP complex *in vivo* and *in vitro*. ***Conclusion:*** C20 plays importantly reno-protective roles most likely through the relocation of CREB-CBP complex.

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Introduction

Traditional Chinese medicine (TCM) has been widely used for the treatment and management of diabetic nephropathy (DN) with few side effects in China. Riligustilide (C20), a natural small molecule, having the structure $C_{24}H_{28}O_4$ and molecular weight of 380.483, was originally isolated from the Chinese herb Ligusticumporteri, a rhizome. Notably, it was reported that riligustilide had neuroprotective effects and was cytotoxic against cancer [1, 2]. Additionally, it has been shown to display competitive binding capability to the GABAa

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receptor, which is a central receptor of inhibitory neurotransmitter [3, 4]. Furthermore, riligustilide also exhibits a potent and specific progesterone-like activity [1, 5]. However, the biological functions about this compound still remains largely unknown [6].

Renal injury is the most common cause leading to end-stage of kidney disease with high mortality. The cause of renal injury is usually directly related to the overload of extracellular factors, such as renin and angiotensin II (Ang II) [7, 8], which may play a major role in the development of renal end-stage [9, 10]. The characteristics of renal injury include hyperfiltration, albuminuria, glomerulosclerosis, glomerular and tubuloepithelial hypertrophy, followed by nephron and potocyte dysfunction leading eventually to kidney failure. The key steps in preventing renal injury are to rescue the damaged renal unit and protect the remained nephronic function by blocking the renin-angiotensin system [11, 12].

The rennin–angiotensin system (RAS) blockers, such as ngiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs), provide renal-protective effects by reducing the amount of reactive oxygen species, mesangial expansion, and proteinuria [13, 14]. Nevertheless, the applications of ACEIs and ARBs are usually clinically limited in use because of their side effects, such as elevation of renin by negative feedback [15, 16]. Aliskiren, a direct renin inhibitor, effectively blocks the RAS activity [14], especially in combination with ACEIs or ARBs [17]. However, recent evidence also suggests that the usage of aliskiren may also cause an increased renin level potentially leading to the incidence of kidney dysfunctions [18]. Consequently, given the drawbacks of the current RAS blockers, exploring novel agents by targeting renin or (pro) renin is urgently required for DN treatments [19].

The cAMP response element binding protein(CREB) is a ubiquitously expressed nuclear transcription factor, which binds with CREB binding protein (CBP) to form a CREB–CBP complex [20, 21]. CBP acts by recruiting CREB at the promoter region of the target gene, thereby regulating gene expression. Studies have suggested that CBP-CREB complex plays a fundamental role in many physiological and pathological processes, including the regulation of glycometabolic genes [22] and RAS [23]. CBP-CREB complex can be stimulated by different extracellular stimuli or small molecules, then get involved in blood glucose control [24, 25]. Several CREB binding sites have been identified in the renin promoter region of the human and mouse, which might indicate a central role of CREB in renin gene expression [26]. It was reported that the level of renin mRNA was decreased by 50% with single knock-down of CREB or CBP, and by 70% with double knockdown of CREB and CBP in cultured cells [26-28], suggesting that the CREB recruited co-activator CBP might be involved in renin biosynthesis and release [29, 30]. Here, our research was to investigate the reno-protective effect of C20 on animal models. Our data supports the notion that C20 plays a role by down-regulating the rennin expression and anti-hyperglycemia effect through CREB-CBP complex in three different animal models.

Materials and Methods

Chemicals

Purification (>99%) and preparation of C20 was carried out from Ligusticumporteri rhizome. Five kilograms of L. porteri rhizome was crushed three times with 95% ethanol. The three crushed parts of extracts with ethanol were combined together and mixed up in 45°C water up to a total volume of 2.5 L, and then repeated three times with 2.5 L of ethyl acetate. The combined ethyl acetate extracts yielded Riligustilide about 200 g after the vacuum concentration at 45°C. Electrospray ionization (ESI) mass spectrometry, nuclear magnetic resonance spectroscopy (NMR), and ^{13}C spectroscopy were used to identify the obtained products.

1mg purified C20 was dissolved in 50 μL of ethanol plus 60% propylene glycol in a total volume of 950 μL ready to use.

Animal studies

All animals were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and were housed at 22°C–25°C under a 12-h light/dark cycle. Animals had free access to food and tap water. Laboratory animals and protocols were approved by Shengjing Hospital of China Medical University, Shenyang, China. The approval number was 2017PS267K. We confirmed that all procedures were conducted according to the guidelines established by Shengjing Hospital of China Medical University, and every effort was made to minimize suffering. Three animal models were employed in the experiments. (1) Eight-week-old male spontaneously hypertensive rats (SHR) were used as the hypertensive kidney model. (2). The Black and Tan with the leptin-deficiency mutation (BTBR ob/ob) obese mice (aged 7–8 weeks-old) were used as the diabetes mellitus type 2 model. The BTBR ob/ob mice carrying the leptin mutation gene may develop hyperglycemia, elevated triglycerides, insulin resistance, mellitus diabetes type 2 and subsequently leading to renal failure. (3). The 5/6 nephrectomized (5/6 NX) rats provided an excellent animal model for chronic renal failure, showing the features of end-stage renal disease IV–V, and presenting the distinction with proteinuria, hypertension and eventually leading to the end stage of kidney. All three animal models were randomly divided into a control group (vehicle; n = 6) and a C20 group (200 μg/kg C20; n = 6). The vehicle solution consisted of ethanol 50 μL and propylene glycol 950 μL. The C20-1 (800 μg/kg for 2 weeks) was intraperitoneally injected into the SHR and 5/6 NX rats respectively. The BTBR ob/ob mice were treated with the 800 μg/kg C20-2 for 20 weeks. The volume of drinking water, blood glucose, urine amount and albumin in urinary were measured after 1 month C20 treatment. The kidneys and hearts were harvested and snap frozen for further analyses.

Cell line

The AS4.1 cell line were cultured in DMEM culture medium containing 5% fetal bovine serum (ReachBio, USA), which derived from glomerular JG cells and highly expressed renin.

Reagents

Mouse anti-CBP monoclonal antibody SC-7300; rabbit anti-CREB polyclonal antibody SC-186 were commercially purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA;). CY5 was obtained from ReachBio WA, USA (#111-165-003), and Alexa Fluor® 488 was acquired from Molecular Probes® (Eugene, OR, USA). And the 4, 6-diamidino-2-phenylindole (DAPI) was gotten from Molecular Probes.

Renin activity assay

Renin activity was determined using the SensoLyte® mouse rennin assay 520 kit purchased from AnaSpec (Fremont, CA, USA), in accordance with the manufacturer's instructions.

Urine analyses

Urine was collected from each rat or mouse 4 hours prior to sacrifice. Urinary albumin, creatinine, and blood urea nitrogen (BUN) were measured using a commercial Elisa kit (Bethyl Laboratories, Montgomery, TX, USA), according to the manufacturer's instructions [31].

Immunohistochemical analysis

The left kidney of individual rats or mice were collected and fixed in fresh 10% formaldehyde and then embedded in paraffin. Sections (3 μm thick) were stained with periodic acid-Schiff (PAS) to evaluate the grade of glomerulo-sclerosis.

Northern blot analyses

Total RNA was isolated from cultured fresh cells and animal tissues using TRIzol® reagent (Invitrogen Life Sciences, Carlsbad, CA, USA). Total RNA (15 mg/ lane) was separated on 1% formaldehyde–agarose gels and transferred onto nylon membranes. The membranes were then hybridized with ^{32}P-labeled renin cDNA probes as described previously [32]. The transcripts were detected by autoradiography and quantified by a Phosphor Imager (Molecular Dynamic, Sunnyvale, CA, USA). The same membranes were then striped and re-hybridized with ^{32}P-labeled 36B4 cDNA probe as the internal loading control.

Luciferase reporter assays

AS 4.1 cells were co-transfected with a luciferase reporter and the pRL-TK plasmid in serum-free media in the presence or absence of C20 using Lipofectamine® 2000 (Invitrogen). The luciferase activity was normalized with β-galactosidase. A pGL4.1k-Luc plasmid with the 4.1 kb DNA sequence in the promoter of Ren-1^c mouse gene was used as the Luciferase reporter as previously described [15, 33].

Electron microscopy

Kidney cortex tissues were fixed in 2% glutaraldehyde and subsequently fixed in1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4. Tissues were then processed as previously described and were observed using a transmission electron microscope [31].

Western blots

Quantification of the protein samples was carried out by western blotting as described previously [34]. Band intensities were quantified by Image J software and normalized to β-actin.

Statistical Analysis

Data are expressed as mean ± SD. An unpaired Student's t test or one-way analysis of variance (ANOVA) was used for statistical comparison in different groups after the demonstration of homogeneity of variance with an F test. A p value less than 0.05 was considered statistically significant.

Results

C20 ameliorated the cardiac and renal symptoms in spontaneously hypertension rats SHR model

C20 reduced the cardiac hypertrophy and renal fibrosis in SHR model, which usually suffered from high blood pressure and then caused cardionephric damage over time. Previous studies have shown that SHR had high serum levels of renin *in vivo*, which usually cause renal damage and other symptoms, such as hyperdipsia, polyuria, proteinuria, and so on [35]. The volume of drinking water (Fig. 1A), urine (mL) (Fig. 1B), and protein urine (Fig. 1C) within 24 h were dramatically decreased with C20 treatment for 6 weeks. In addition, the cell membranes of left ventricular cardiomyocytes stained with FITC-labeled wheat germagglutinin showed a marked atrophic size of cardiomyocytes in response to C20 treatment (Fig. 1D). Significant decrease of blood glucose was found at 15, 30, and 60 min in the C20-treated group in the SHR (Fig. 1E). The renal fibrosis (Fig. 1G) and glomerulus size (Fig. 1F) also showed a noticeable decrease in the C20-treated groups, which were significantly elevated in vehicles. These data suggested that C20 may regulate the function of the heart and kidney.

C20 alleviates the diabetic nephropathy (DN) in BTBR ob/ob mice

BTBR ob/ob animal model is a great model for the study of diabetic nephropathy, which is inclined to lead to the end stage of kidney. Therefore, this model was chosenas one of the C20 research models for study of renal injury. C20 significantly attenuated glomerular fibrosis and atrophy by PAS staining in BTBR ob/ob model (Fig. 2A). The levels of fasting blood glucose were decreased in week 1 and significantly decreased in week 2 after C20 treatment compared to the blood glucose in control mice (Fig. 2B). To further explore the prevention mechanism of C20 underlying the animal from protein urine, the microstructure of podocyte were examined by electron microscopy in our study, since podocyte damage plays a important role in the involvement in albuminuria of diabetic nephropathy. The foot process effacement in podocytes and the glomerular basement membrane thickness in glomeruli were remarkably improved in the C20-treated mice compared to vehicle mice observed by scanning electron microscopy (Fig. 2C). Fibronectin levels in kidney lysates, which was considered as fibrosis marker (Fig. 2D), were also decreased in response to C20 treatment.

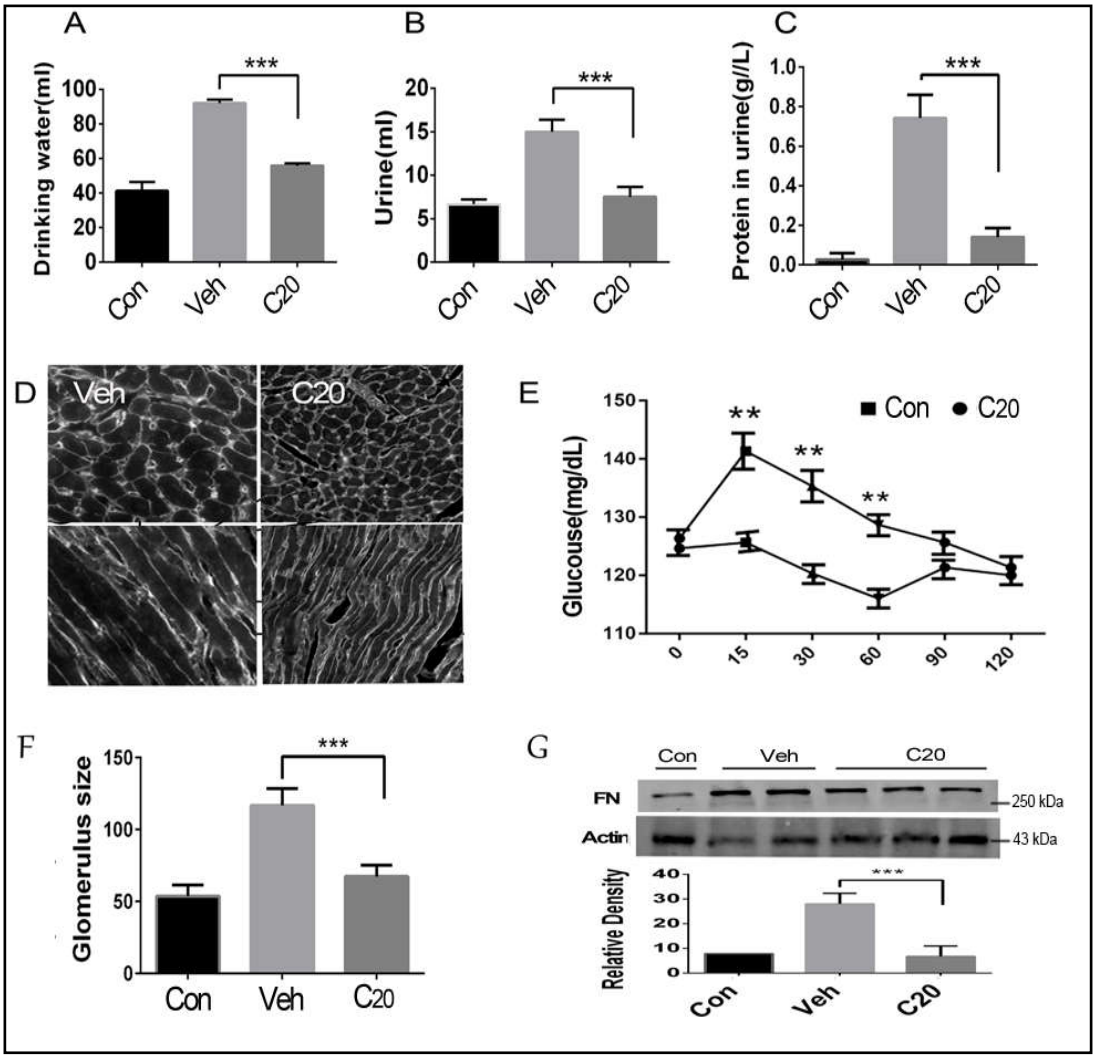

Fig. 1. C20 ameliorated the phenotype of spontaneously hypertensive rats (SHR).The amounts of drinking water (A), urine (mL) (B), and protein uria (C) for 24 hours were dramatically reduced at 6 week after C20 treatment. (D) The sections of left ventricle stained with FITC-labeled wheat germ agglutinin were observed using an electron microscope, demonstrating a remarkable reduction in the sizes of cardiac myocytes in response to C20 treatment. (E) C20 had a regulating effect on glucose tolerance in SHR. The blood glucose levels of SHR treated by C20 were significantly lower than those of the control group at 15, 30, 60 minutes respectively in the glucose tolerance test. ** P<0.01 (F) C20-treated group showed a noticeable decrease in the glomerulus size quantified by image J. (G) Renal western blot analysis using anti-fibronectin antibody indicated that the degree of renal fibrosis was reduced in the C20-treated group compared to the vehicle group. ***P<0.0001.

C20 mitigated nephropathic progress in 5/6 nephrectomy (NX) rats

The 5/6 NX model was used to further investigate the protective effect of C20 on the renal injury in our study. Serum levels of Ca^{2+} (Fig. 3A), parathyroid hormone (Fig. 3B), and phosphorus (Fig. 3C) were not significantly changed in C20 treated rats compared to control group, however the levels of urinary albumin (Fig. 3D) and BUN (Fig. 3E) were noticeably reduced in the C20-treated 5/6 NX rats (Fig. 3F). The 5/6 NX rats developed albuminuria much earlier with more severity in comparison with the C20 treated group for 5 weeks evaluated by the urinary albumin to creatinine ratio (ACR) (Fig. 3F). These data again further demonstrated the protective effect of C20 on renal injury in 5/6 NX rat model.

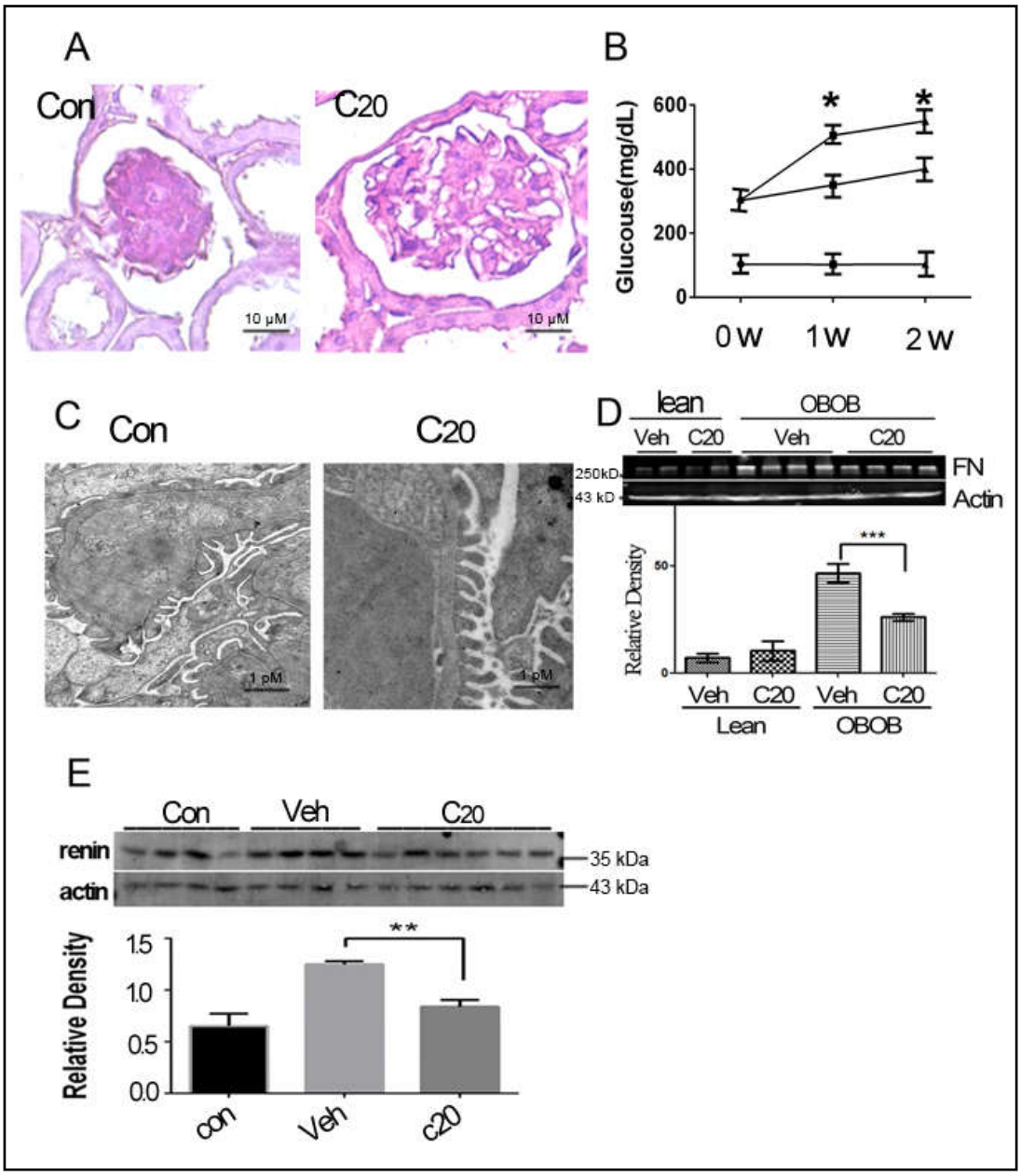

Fig. 2. C20 Attenuated diabetic nephropathy in BTBR ob/ob mice.All mice were sacrificed at 19 weeks subsequent to intervention, and the kidneys were harvested for histological analyses. Representative images of glomerular morphology staining with periodic acid-Schiff (PAS) demonstrated an attenuated glomerular atrophy in the C20-treated group. (B) Fasting blood glucose was significantly decreased at week 1 and week 2 after C20 Challenge, which droped a hint that C20 plays a regulative role in the blood glucose. (C) The glomerular basement membrane and podocytes were observed using a scanning electron microscope, suggesting that the foot process effacement in podocytes and the thickness of glomerular basement membranes were obvious improvement in the presence of C20. (D) (E) The protein levels of fibronectin and renin in kidney lysates were semi-quantified by western blot analyses. The expression of two proteins significantly decreased in the C20-treated group compared to the other groups. *C20 versus vehicle groups; P< 0.05.

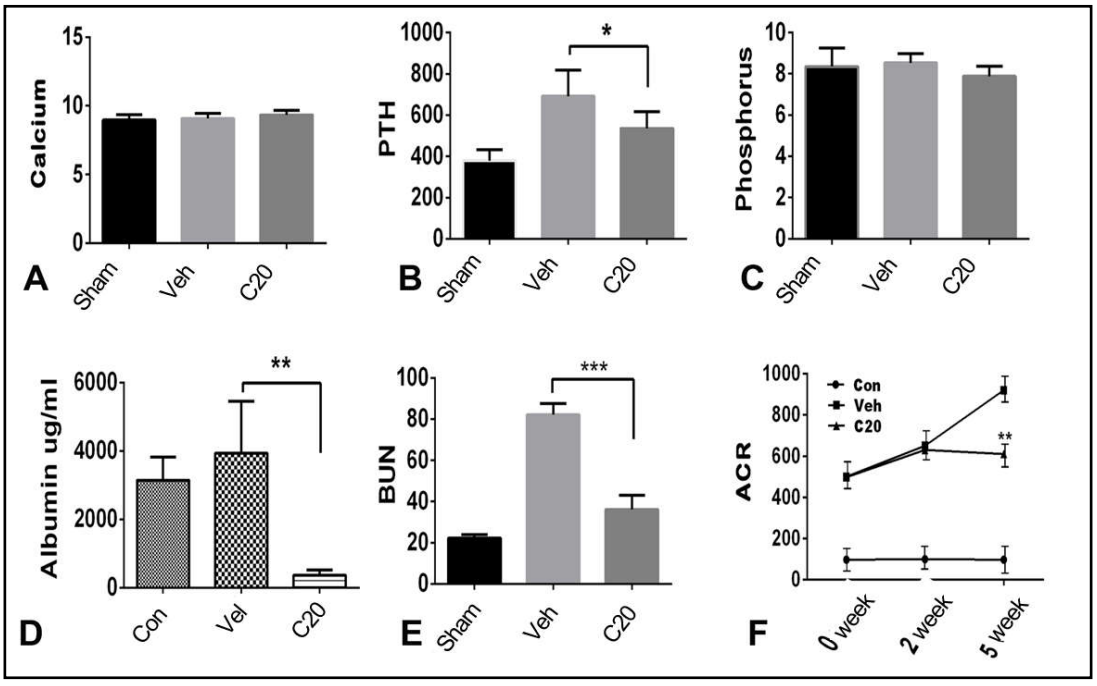

Fig. 3. C20 also significantly alleviated renal injury in 5/6-nephrectomized (5/6 NX) rats. First of all, Ca^{2+} (A), parathyroid hormone (B), and phosphorus (C) in serum were determined to eliminate the interference of these factors. The related indexes of impaired renal function, such as albumin in urine (D), urea nitrogen (BUN) in blood (E) and urinary albumin to creatinine ratio (ACR) (F), were then checked and noticeably reduced in the C20-treated group compared with vehicle group. Our data suggested that with C20-treatment, the rats might delay the occurrence of renal dysfunction due to nephrectomy. $^{*}P< 0.05$; n = 5–7 rats in each group.

C20 down regulated renin expression in vivo and in vitro

In order to further study the molecular mechanism of C20 underlying the renal protection, the expression of renin were also examined *in vivo* and *in vitro*, which was a major pathogenic factor to promote renal injury. Renin expression was remarkably reduced in the C20-treated group in the BTBR ob/ob mice with Western blotting (Fig. 2E). A total of 20 compounds extracted from Chuanqiong were examined, of which only C20 was found to significantly decrease the renin expression. Vitamin D served as a positive control (Fig. 4A, B) because previous studies have shown that vitamin D can significantly reduce the expression of renin [36]. Luciferase assays were used to evaluate the C20 effect on the renin promoter by transfected fragment of renin promoter into the AS 4.1 cells. The dose dependent manner was found in the luciferase experiment in response to the C20 challenge animal (Fig. 4E). The results of the renin activity showed a time-manner decrease both *in vitro* (Fig. 4C) and *in vivo* in response to C20 (Fig. 4D). These data repeatedly proved that C20 may significantly regulate the expression of renin.

Effect of C20 on the expression of renin, CBP, and CREB in renal JG cells

Many studies have shown that the CBP-CREB complex regulates the expression of rennin [27], therefore we have paid special attention to the expression of this complex in this study. Renin, CBP, and CREB were detected by immuno-fluorescent staining JG cells in kidney in SHR. The expression levels of renin, CBP, and CREB were dramatically decreased in a therapeutic dose-dependent manner in response to C20 treatment (Fig. 5).The expression levels of renin and CBP-CREB were reduced in C20 treatment group from the dose of 0.4 mg, and the positive immunostaining of the two proteins was barely seen at the C20 dose of 8 mg. Representative immunofluorescent images suggested that CBP and CREB trans-located from

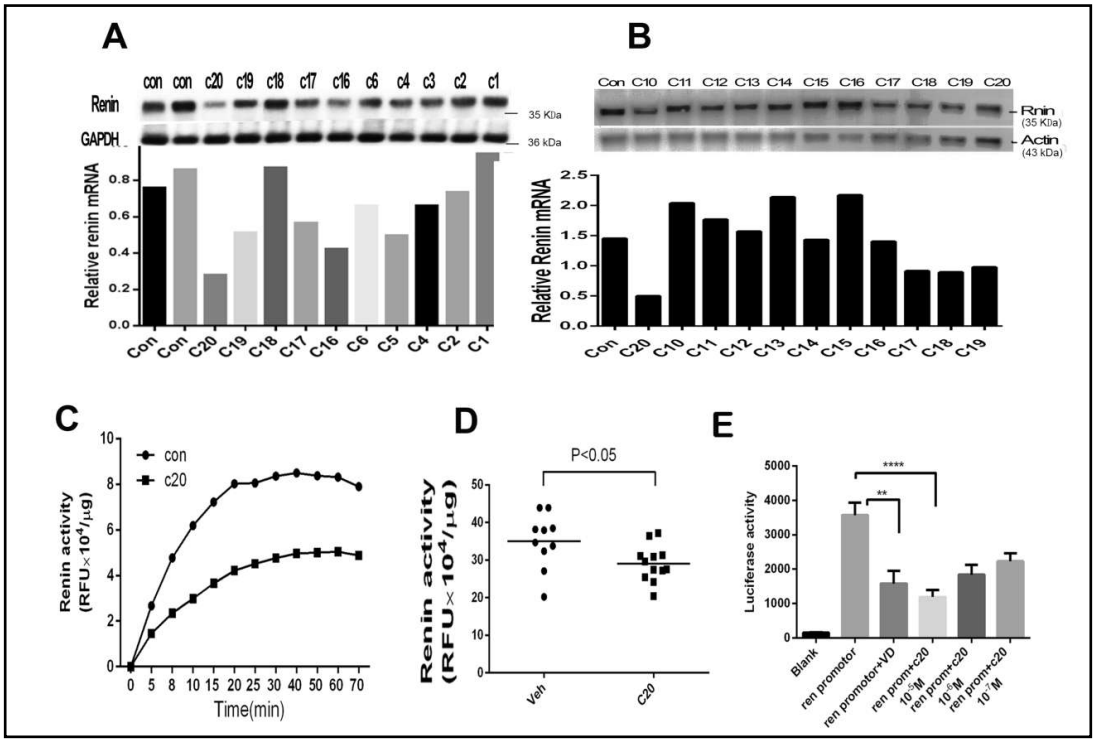

Fig. 4. C20 targeted the down regulation of renin expression in vivo and in vitro. (A) (B) The semi-quantification analyses of renin mRNA were performed to select compound targeting to the expression of renin by Northern blots. A total of 20 compounds were extracted from Chuanqiong, of which only twentieth of the 20 compounds (that is C20) had the dramatical function to reduce the expression of renin in AS 4.1 cell line, using vitamin D as a positive control. (C) The renin activity curve in respond to one shot of C20 was detected in AS4.1 cells by a Sensolyte 520 mouse renin assay kit. (D) Renin activity was measured in group vehicle and C20, respectively. Data showed that C20 can significantly reduce renin activity in SHR, $p<0.05$. (E) Luciferase assays were used to evaluate the regulation of renin by C20 through a renin promoter segment transfected into AS4.1 cells. C20 versus vehicle*$P< 0.05$; n = 5–7 rats in each group.

the nucleus to the cytoplasm with C20 treatment over time in AS4.1 cells (Fig. 6). Because many studies showed that renin, CBP, and CREB were observed to be co expressed in JG cells, there might be a crosstalk among rennin, CBP and CREB [26, 27, 33, 37].

To explore this problem, a software was used to predict whether two transcriptional factors have possible binding sites with C20. A molecular docking study was carried out by the MOE (Molecular Operating Environment, version 2015.1001) software with the default settings. According to this model, the C20 was inserted into the long-narrow triangle cavity of the binding pocket in CBP protein. CBP crystal structure obtained from Protein Data Bank (PDB ID: 2D82) was employed as the protein template in calculation. A molecular simulation of C20 into the binding pocket of CBP was detected, which may predict the interactions of small molecule with protein. Additionally, the C20 accepted a hydrogen bond from the hydroxyl of Gln A1113 in CBP, which made them hold more tightly (Fig. 7).

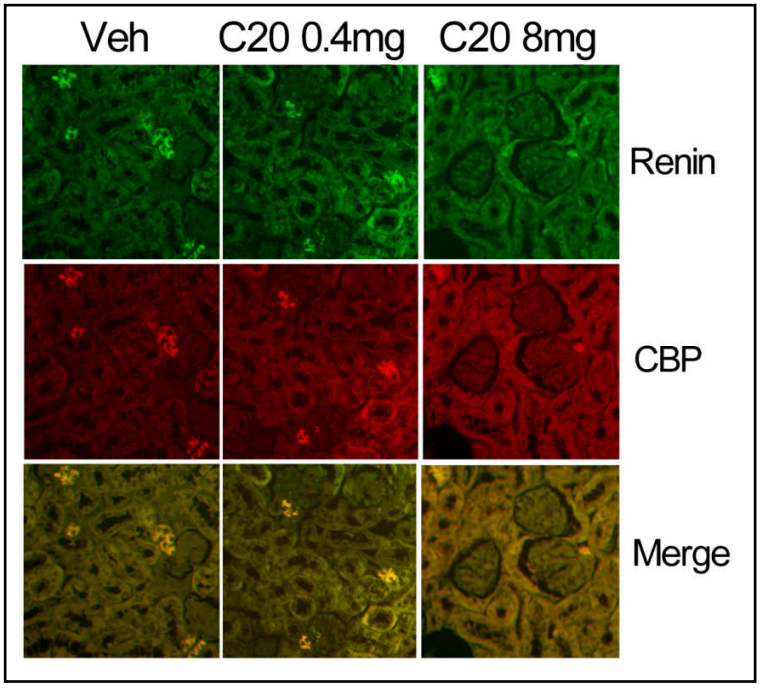

Fig. 5. C20 affects the expression of renin, CREB binding protein (CBP), and cAMP response element binding protein (CREB) in renal JG cells. Renin, CBP and CREB were examined by immune fluorescent staining at 1 week following high salt challenge in JG cells of kidney. The expression of renin, CBP, and CREB were decreased in response to C20 in a therapeutic dose-dependent manner. The effective doses of C20 to reduce the expression of rennin and CBP-CREB are from 0.4 mg/kg to 8 mg/kg. Renin, CBP, and CREB were found co-location in JG cells of kidney.

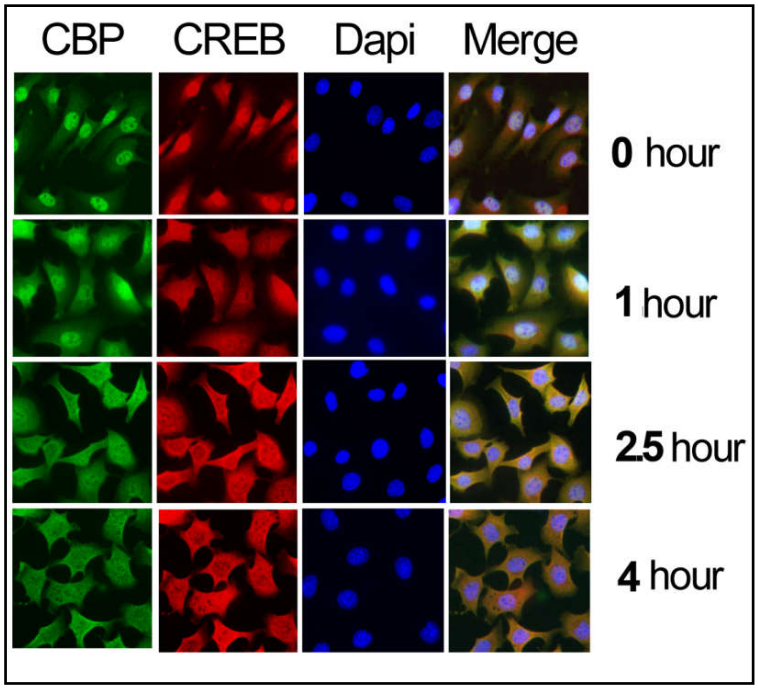

Fig. 6. C20 effected the expression on the of renin, CBP, and CREB in time course manner in AS4.1 cells. Representative images of immunofluorescent staining suggested that CBP and CREB were translocated from the nucleus into the cytoplasm after treatment with C20 over time.

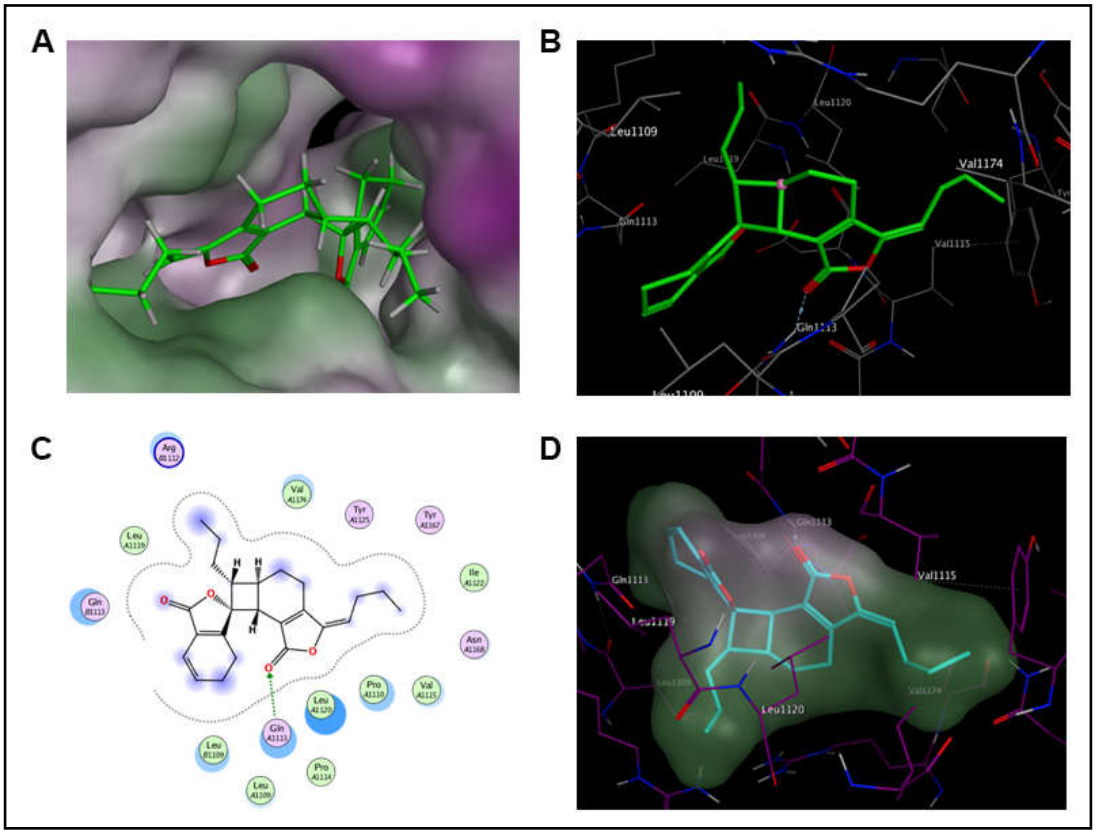

Fig. 7. Docking pose of compound C20 (green) within the protein binding pocket. To rationalize the observed binding model of C20 with CBP, docking simulations of C20 in the CBP binding pocket were performed. The crystal structure of a CBP complex (PDB code 2D82) was used as the protein template (A).The docking model (B) showed that the molecular of C20 very closely overlapped with the original ligand of CBP (B,C). (D) The merged image of the CBP complex structure.

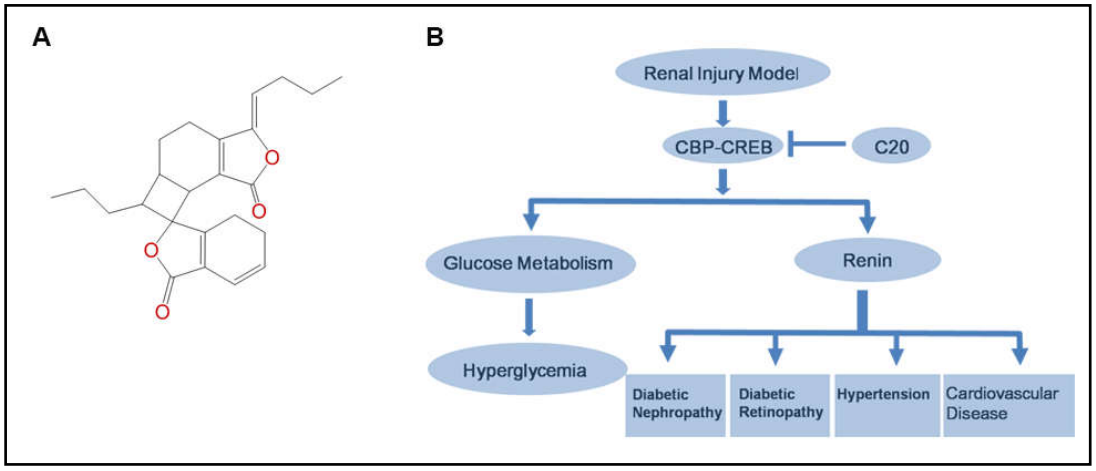

Fig. 8. The profile of C20 and the proposed mechanism model. (A) The molecular formula of C20. (B) The mechanistic perspicacity for C20 to mediate CBP-CREB on the renin pathways in kidney protection and on mitigating hyperglycemia has been depicted. The solid line shows speculation.

Discussion

Our previous studies have shown that the levels of serum renin are high in SHR [38] and diabetic mice [31]. Renal mass cut led to hyperfiltration and augment of intraglomerular pressure in residual glomeruli (Fig. 3). Therefore, increased oxygen consumption and work burden in hypertrophic nephrons (Fig. 1, F) can give rise to activated renin signaling system and result eventually in glomerulosclerosis and tubulointerstitial fibrosis [39]. It has been shown that the degree of renal injury is positive association with the activity of renin [36]. To explore the reno-protective role of C20 in this study, the three renal damage models were used, which are characterized by the kidney injury and high levels of the renin [36, 38].

Many scholars have suggested that renin may be involved in the initiative and accelerative progression of renal damage and cardiac hypertrophy in animal models [40]. Studies have demonstrated that high level of renin might be associated with many diseases, therefore, targeted reduction of renin expression is essential to the treatment of these diseases. Nevertheless, to our knowledge, previous studies have focused on the blockade of the renin-angiotensin pathway, this is the first study aimed at directly decreasing renin expression to treat or prevent diabetic nephropathy and renal hypertension. In the present study, we found that C20 can alleviate the renal damage by targeting down-regulation of renin expression in the SHR (Fig. 1), the ob/ob obese mouse (Fig. 2), and the 5/6 NX rat models (Fig. 3). We further demonstrated that C20 had beneficial effects on the prevention of glomerular dysfunction as characterized by improved proteinuria and podocyte morphology in our animal models. Our data also showed that the diabetic mice with C20 treatment dramatically reduced the expression of fibronectin (Fig. 2), which is considered as a biomarker of fibrosis in the kidney [41]. Moreover, we have demonstrated that C20 suppressed renin expression under normal levels of calcium and phosphorus *in vivo*, which further indicated the beneficial role of C20 on renal protection. The down-regulation of renin expression markedly ameliorated the renal and cardiomyocyte injury [39, 42].

Renin is synthesized and released from JG cells at the afferent arteriole in the glomerulus where CREB/CBP complex co-locates. CBP and CREB are required for the expression of renin and maintenance of the function of JG cells in the kidney [37]. In this study, immune fluorescence staining was used to determine the translocation of the CREB/CBP complex from the nucleus to the cytoplasm with C20 treatment. Basically, CBP and CREB were mainly found in the nucleus at the basal statue in AS4.1 cells in our study. Interestingly, it was also found that renin and CREB/CBP complexes were co-localized in the juxtaglomerular cells, which were suppressed by C20 challenge in a dose-dependent manner in our study. Additionally, to investigate the effect of C20 on CBP/CREB (PDB ID: 4NR4) complexes, we used MOE software to predict the possibility of binding sites between C20 and these two proteins respectively. We found that only the combination of C20 to CBP was relatively tight in MOE model (PDB ID: 2D82), which further indicated that C20 might have the interaction of molecule-protein with CREB-CBP complex (Fig. 7).

Many studies address CBP-CREB complex plays a role in responses to blood glucose and energy balance. Studies have also indicated CBP as the most connected factor in protein-protein interactions in diabetes [43]. Accidentally there was a significant regulatory role of C20 on hyperglycemia at a higher dose in our two animal models, namely SHR and OB/OB mice. We assume that C20 plays a role through the CBP complex that regulates blood glucose and controls renin.

Conclusion

Collectively, our data suggested that C20 reduced the activity of renin through the relocation of CREB–CBP transcriptional complex, which downstream mediated the renin gene and metabolic-related genes. Whereas C20 may exerted a pronounced therapeutic effect on nephropathy related to renin. As a promising compound, C20 may be useful in preventing

and/or treating diabetes, diabetic nephropathy and cardiac hypertrophy most possibly though the CBP-CREB complex. Therefore, the proposed mechanism of C20 whereby CBP-CREB complex governs the renin pathway to alleviate kidney injury and governs the glucose metabolism gene to inhibit hyperglycemia was shown in Fig. 8.

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Disclosure Statement

The authors declare that no conflicts of interest exist.

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