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Original Article

Breviscapine attenuatted contrast medium-induced nephropathy via PKC/Akt/MAPK signalling in diabetic mice

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Abstract: Contrast medium-induced nephropathy (CIN) remains a major cause of iatrogenic, drug-induced renal injury. Recent studies reveal that Breviscapine can ameliorate diabetic nephropathy in mice. Yet it remains unknown if Breviscapine could reduce CIN in diabetic mice. In this study, male C57/BL6J mice were randomly divided into 7 groups: control, diabetes mellitus, CIN, diabetes mellitus+CIN, diabetes mellitus+Breviscapine, CIN+Breviscapine and diabetes mellitus+CIN+Breviscapine. Model of CIN was induced by tail intravenous administration of iopromide and model of diabetes mellitus was induced by Streptozotocin intraperitoneally. Breviscapine was administered intragastrically for 4 weeks. Renal function parameters, kidney histology, markers of renal fibrosis, phosphorylation of protein kinase C/Akt/mitogen activated protein kinases were measured by western blot. We found out that diabetes mellitus aggravated CIN damage. Renal histological analysis showed Breviscapine reduced of renal fibrosis and tubular damage. Breviscapine was also shown markedly to ameliorate CIN fibrotic markers expression, reduced proteinuria and serum creatinine. Furthermore, Breviscapine decreased phosphorylation of PKCβII, Akt, JNK1/2 and p38. Therefore, Breviscapine treatment could ameliorate the development of CIN in diabetic mice, which was partly attributed to its suppression of renal fibrosis via phosphorylation of PKCβII/Akt/JNK1/2/p38 signalling.

Keywords: Contrast medium-induced nephropathy, diabetes mellitus, breviscapine, PKC/Akt/MAPK signalling

Introduction

Contrast medium (CM)-induced nephropathy (CIN) after clinical practice is association with significant in-hospital, morbidity and mortality in patients with coronary angiography and intervention [1-3]. In recent studies, CIN is characterized by increased permeability to proteinuria [4], up-regulation of serum creatinine [4] and excessive synthesis of extracellular matrix (ECM) [5-7], including collagen I, connective tissue growth factor (CTGF) and transforming growth factor β1 (TGF β1), eventually resulting in renal impairment. Meanwhile, Breviscapine can improve diabetic nephropathy obviously in previous studies [8, 9]. However, it remains unknown if Breviscapine can reduce CIN impairment in diabetic mice.

With the increase use of CM in diagnostic and interventional procedures in clinical work, CIN has become a main reason of renal injury [10], accounting for a large part of all kidney failure cases [11-14]. Patients at higher risk include those with baseline diabetes mellitus. Individual risk factors that predict CIN have been identified and cumulative risk developed. Effort to modify the risk of CIN have included the investigation of variously preventive hydration strategies [15], various types of contrast medium, and most recently pretreatment of preventive medicine with unknown conflicting results, such as Breviscapine [9, 16]. Mostly likely CIN is a multifactorial phenomenon [17]. The pathogenesis of CIN in diabetic mice is still poorly understood.

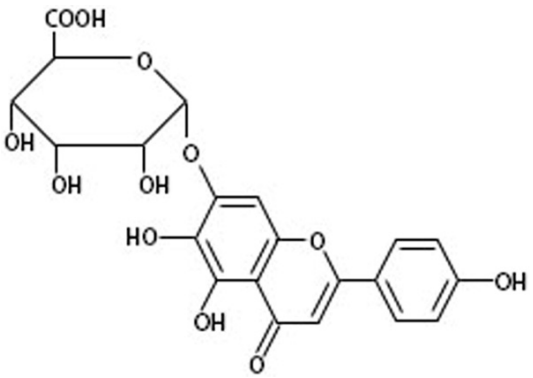

Figure 1. The chemical structure of Breviscapine.

Breviscapine is a flavonoid extracted from the Chinese herb *Erigeron breviscapus* (Vant), Hand Mazz, and prepared into a Chinese patent medicine [18, 19]. Its essential active ingredient is 4'-hydorxyscutellarin 7-O-glucuronide, which is shown as (**Figure 1**). As far as we know, Breviscapine's variously pharmacological action is dilating micrangium, reducing blood viscosity, improving microcirculation and down-regulating ECM acculation, especially as an anti-protein kinase C (PKC) inhibitor [20-22]. Some prior researchers have studied whether and how Breviscapine can prevent diabetic nephropathy [22], but little is known about the influence of Breviscapine on CIN in diabetic mice and its underlying mechanisms.

ECM synthesis and many signalling pathway are related both in heart and kidney in diabetic mice [9, 18, 23, 24]. The kidneys of experimental diabetic animals exhibit increased ECM generation, PKC, Akt and MAPK activition, which, moreover, could trigger renal injury. PKC activation, especially the PKCβII isoform, has been reported to be selectively related with activation of Akt in diabetic animals [25, 26]. We have characterized the effects of PKC activation on multiple steps of Akt phosphorylation, which is partially responsive to PKC activation in diabetes mellitus. Akt regulates a number of cellular functions, including glucose metabolism, protein synthesis, and cell fibrosis [27-30]. In the case of diabetic nephropathy, long-term hyperglycemia regulates the Akt activation and contributes to enhanced p38 MAPK activation [30]. In addition, Akt has been identified as an important gene for maintenance of normal glucose homeostasis in diabetic mice [31]. Moreover, Akt has been investigated for its role in hyperglycemia-induce diabetic nephropathy. Three main MAPK subfamilies have been well characterized: ERK1/2, JNK, p38. Each of these MAPK subfamilies is shown to be involved in the pathogenesis of CIN and diabetes mellitus [32]. However, contributions of the PKCβII/Akt/MAPK signalling to CIN in type 1 diabetes mellitus have not received much attention.

Previous studies reported that Breviscapine could lower proteinuria and improve serum creatinine abnormalities in diabetic nephropathy, possibly by reducing renal injury [16]. Breviscapine has been proved to improve kidney function in diabetic mice. However, it is unknown whether Breviscapine can also act by these effects and mechanisms in CIN in diabetic mice.

In this study, we aimed to evaluate the renal injury in CIN in diabetic mice. Then, we assessed the effect of Breviscapine on kidney function, and studied the signalling pathway involving PKCβII/Akt/MAPK in CIN in Streptozocin (STZ) -induced diabetic mice.

Materials and methods

Animals

Eight-week-old male C57/BL6J mice were purchased from Zhejiang University experimental animal center and housed in a pathogen-free laboratory at Sir Run Run Shaw Hospital, medicine of college, Zhejiang University. The study was conducted in accordance with the guidelines and requirements from the Guide for the Care and Use of Laboratory Animals (NIH Publication, 8th Edition, 2011) and the Institutional Animal Care and Use Committee of Zhejiang University. All experimental protocols were approved by the Ethics Committee of Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University.

Models of CIN in diabetic mice

In this study, male C57/BL6J mice were randomly divided into 7 groups: control, diabetes mellitus (D), CIN, diabetes mellitus+CIN (DC), diabetes mellitus+Breviscapine (DB), CIN+Breviscapine (CIN+B) and diabetes mellitus+CIN+Breviscapine (DCB). Mice were rendered diabetes by intraperitoneal in injection of STZ 50 mg/kg on 5 consecutive days, whereas

other mice were injected with vehicle (citrate buffer, pH 4.5) [33-35]. Each group had 5-7 mice. 5-7 days after the injection, blood glucose was tested by applying tail vein blood to a glucometer as previously described [33]. Hyperglycemia was defined as a random blood glucose level of > 16.7 mmol/L [33]. After the model of diabetes mellitus was established, mice were prohibited drinking water for one night, and then mice were injected at a dose of 10 ml/kg iopromide via tail vein administration over the course of 1 minute. Then the mice were treated intragastrically with or without Breviscapine (10 mg/kg/d) for 4 weeks [23]. Other mice groups except for Breviscapine treatment groups (DB, DCB and CIN+B) were administered the same volume of phosphate buffered solution intragastrically for 4 weeks. Then mice were sacrificed by an intraperitoneal injection of chloral hydrate.

Parameters of renal function

Twenty-four hours urine in mice was collected and blood sample were taken from eyeballs in sacrificed mice. EDTA (1.5 mg/ml) was added to the blood to impair coagulation. Immediately after the sacrifice, both kidneys were respectively preserved in liquid nitrogen and 10% formaldehyde. Kidneys preserved in liquid nitrogen were tested in protein and RNA levels. Kidneys were immersed in 10% formaldehyde for 24 hours and then embedded in paraffin. Adjacent 4 μm sections were stained with hematoxylin and eosin (H&E), Masson trichrome staining, Periodic Acid-Schiff (PAS) staining, periodic acid-sliver methenamine (PASM) staining. Proteinuria and serum creatinine levels were measured by a standard spectrophotometric assay (Jiancheng, Nanjing). Two independent observers examined the histological findings. The pathological score was as follows: 0 = no damage; 1 = minimal injury; 2 = moderate injury; 3 = intermediate injury; 4 = severe injury. Ten microscope fields were analyzed in each section from each mice.

RNA extraction and quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) from kidneys tissue of experimental mice using a standard protocol [36]. cDNA synthesis was performed with 1 μg of total RNA using the miScript II RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. qRT-PCR and data analysis were performed with the ABI 7500 cycler (Applied Biosystems, CA, USA). β-actin was used as the endogenous control for mRNA expression. The primers that we designed were as follows: collagen I forward, 5'-GAGCCTAACCATCTGGCATCT-3', reverse, 5'-AGAACGAGGTAGTCTTTCAGCAAC-3'; CTGF, forward, 5'-CAGGGAGTAAGGGACACGA-3', reverse, 5'-ACAGCAGTTAGGAACCCAGAT-3'; TGF β1 forward, 5'-GTGTGGAGCAACATGTGGAACTCTA-3', reverse, 5'-TTGGTTCAGCCACTGCCGTA-3'; β-actin, forward, 5'-TCATCACTATTGGCAACGAGC-3, reverse, 5'-AACAGTCCGCCTAGAAGCAC-3'.

Western blots

Total protein from kidneys were extracted in a RIPA lysis buffer (Beyotime, Shanghai, China), which was supplemented with 1 mmol/L PMSF [36]. Protein concentrations were determined using a BCA assay kit (Beyotime, Shanghai, China). Equal amounts of protein (40 μg) were separated on 10% sodium dodecyl sulphate polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% non-fat milk-TBST and incubated overnight with primary antibodies at 4°C, followed by 1 hour of incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature. The bands were visualised with an enhanced chemiluminescence reagent (Amersham, Haemek, Israel) on a LAS-4000 image reader system (Fujifilm, Tokyo, Japan). To ensure equal protein loading, the β-actin protein was used as the endogenous control.

The anti-collagen I, anti-CTGF and anti-TGF β1 antibodies were purchased from Abcam Public Limited Company (Abcam, Cambridge, UK). The anti-β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other antibodies were purchased from Cell signalling Technology (CST, USA).

Statistics

All experiments were performed at least three times. The data were presented as the mean ± standard error of the mean (SEM). Statistical analysis was conducted with SPSS 20.0 software, using one-way ANOVA for multiple group

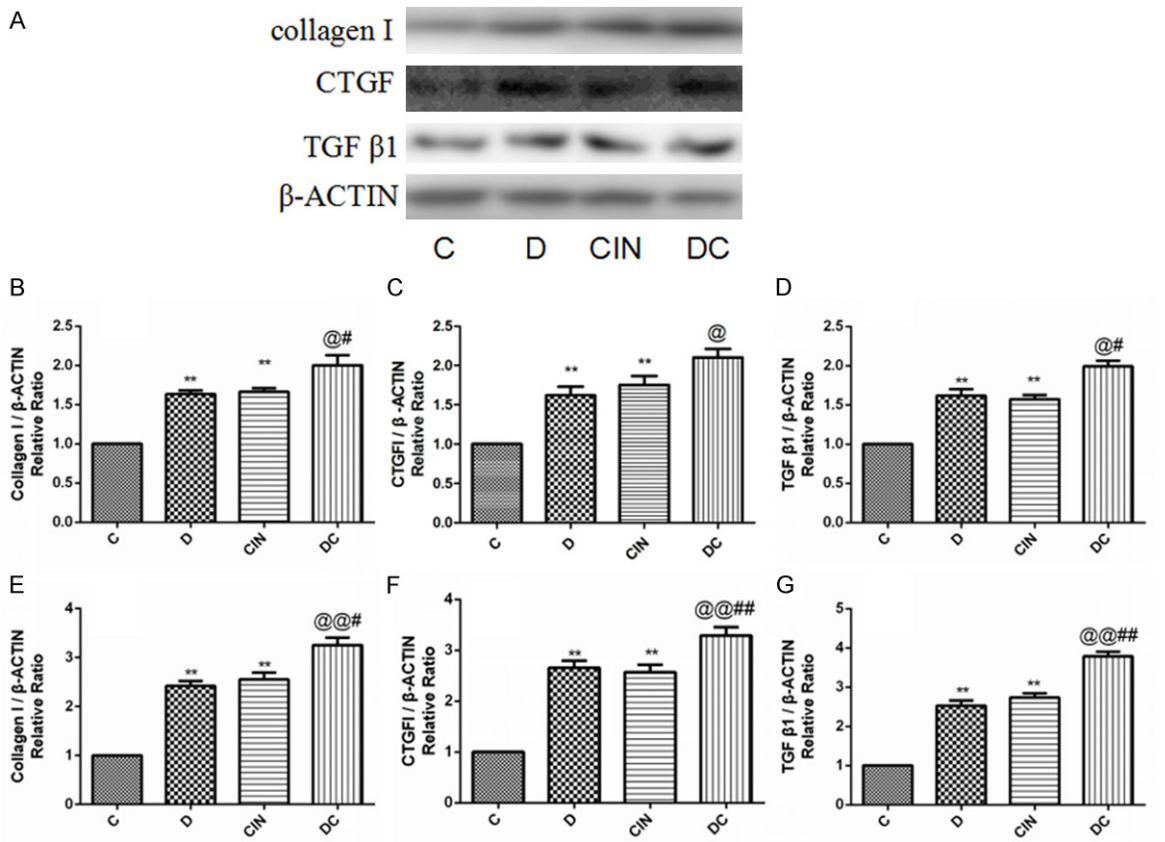

Figure 2. Expression of ECM in model of CIN in diabetic mice. A. Western blot of collagen I, CTGF, TGF β1 proteins expression in CIN of diabetic mice. B-D. Relative protein expression levels of collagen I, CTGF and TGF β1 are normalised to β-actin and expressed as the mean ± SEM, n = 5. $^{**}p < 0.01$ vs. C group. $^{@}p < 0.05$ vs. D group. $^{\#}p < 0.05$ vs. CIN group. E-G. Relative mRNA expression levels of collagen I, CTGF and TGF β1 are normalised to β-actin and expressed as the mean ± SEM, n = 5. $^{**}p < 0.01$ vs. C group. $^{@@}p < 0.01$ vs. D group. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. CIN group.

comparisons or Student's *t* test for two-group comparisons. $P < 0.05$ was considered to be statistically significant.

Results

Expression of collagen I, CTGF, TGF β1 in model of CIN in diabetic mice

The model of CIN in diabetic mice was established in C57/BL6J mice that were treated with STZ and iopromide, and age-matched controls were included. When compared with control group, the protein level of collagen I was enriched to approximately 1.6/1.7/1.9-fold (**Figure 2A**, **2B**) and the mRNA level of collagen I was at approximately 2.4/2.6/3.2-fold (**Figure 2E**) in D/CIN/DC group; the protein level of CTGF was up-regulated to approximately 1.6/1.8/2.0-fold (**Figure 2A**, **2C**) and the mRNA level of CTGF was at approximately 2.7/2.6/3.2-fold (**Figure 2F**) in D/DB/DC group; the protein level of TGF β1 was enriched to approximately 1.6/1.5/1.9-fold (**Figure 2A**, **2D**) and the mRNA level of TGF β1 was at approximately 2.5/2.7/3.7-fold (**Figure 2G**) in D/DB/DC group.

Taken together, the above results strongly suggested that ECM up-regulation played an important role in model of CIN in diabetic mice.

Decreased renal injury in CIN in diabetic mice treated with Breviscapine

Morphological changes in both the glomerular and tubulointerstitial compartments, including tubular atrophy and interstitial fibrosis, were highlighted by hematoxylin-eosin staining (H&E) (**Figure 3A**, **3E**) and Masson trichrome staining (**Figure 3B**, **3F**). PAS (**Figure 3C**, **3G**) and PASM (**Figure 3D**, **3H**) staining showed tubular glycogen and glomerular mesangial matrix accumu-

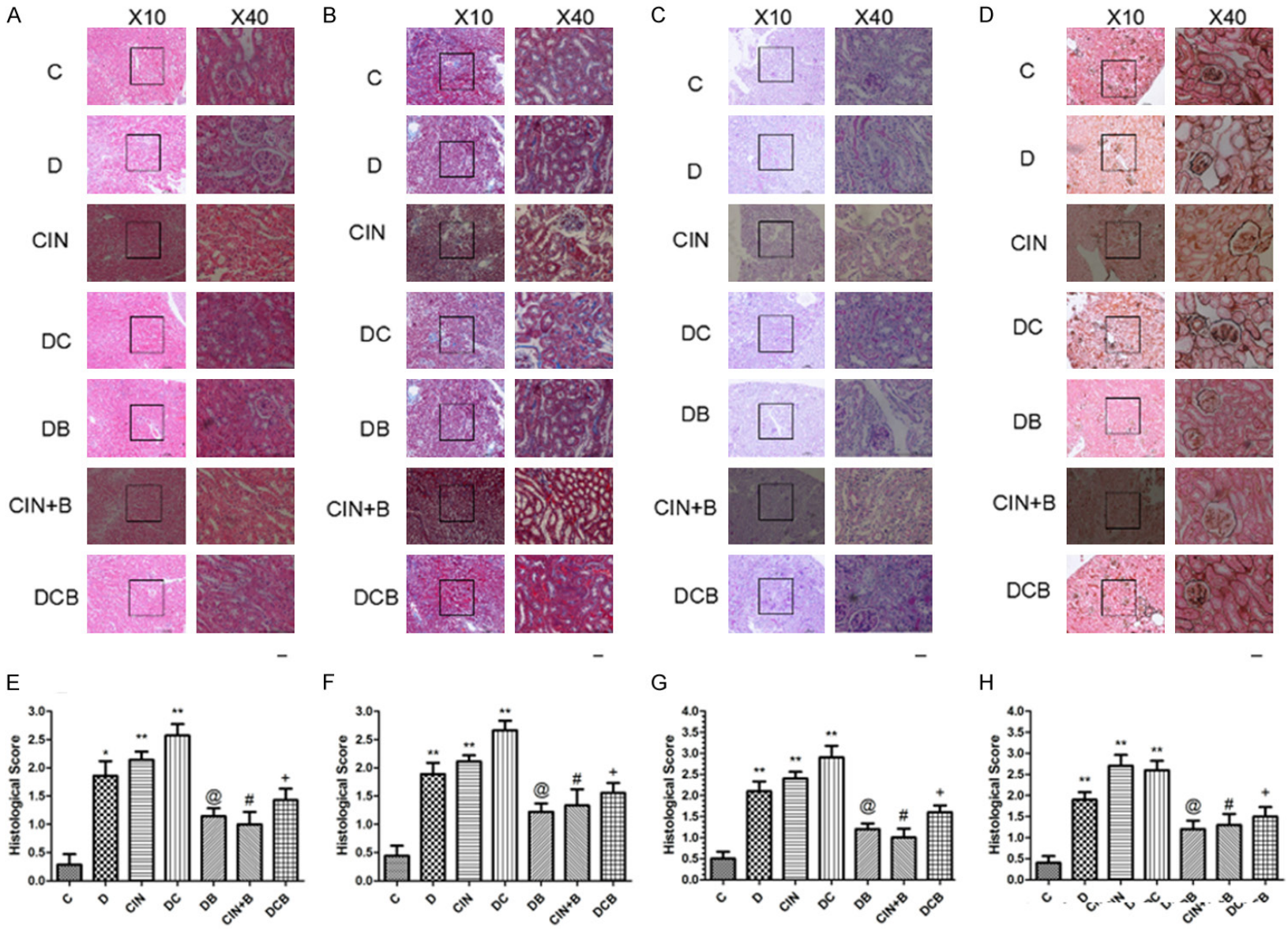

Figure 3. Histological observation of Breviscapine-treated CIN in diabetic mice. H&E (A) showed renal atrophy and Masson trichrome staining (B) showed renal fibrosis. PAS staining (C) and PASM staining (D) respectively showed glycogen accumulation and injury of glomerular capillary basement membrane at magnification

x 10 and x 40. Scale bar: 50 μm. Quantification of H&E, Masson trichrome staining, PAS and PASM was shown as (E-H) respectively. n = 5. $^{**}p < 0.01$ and $^{*}p < 0.05$ vs. C group. $^{@}p < 0.05$ vs. D group. $^{\#}p < 0.05$ vs. CIN group. $^{+}p < 0.05$ vs. DC group.

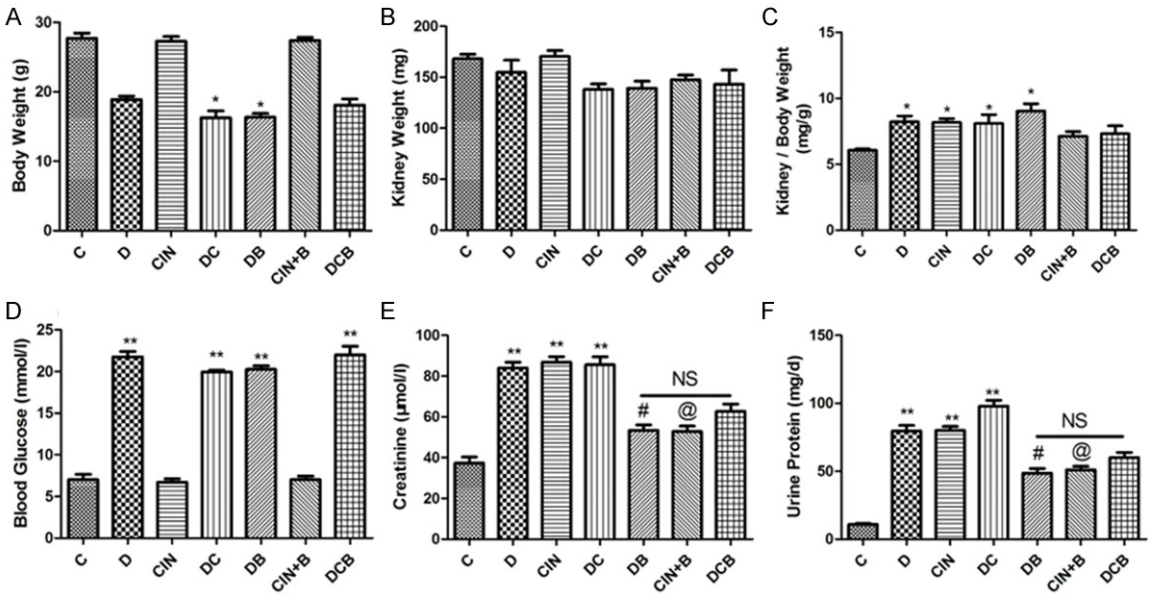

Figure 4. Metabolic and physiology parameters in experimental mice. Body weight (A), kidney weight (B), body/kidney weight ratio (C), blood glucose (D), serum creatinine (E) and proteinuria (F) in experimental mice were shown in the figure. n = 5. $^{**}p < 0.01$ and $^{*}p < 0.05$ vs. C group. $^{\#}p < 0.05$ vs. D group. $^{@}p < 0.05$ vs. CIN group. No significance (NS) for DB, CIN+B and DCB group.

lation in kidney specimen of CIN in diabetic mice. PAS staining was used to detect the glycogen accumulation in the kidney tissues. PASM staining was used to observe the morphological changes of glomerular capillary basement membrane in the damage, such as rupture, hyperplasia, folding and so on.

In D, CIN and DC groups, tubular atrophy and fibrosis areas were more obvious compared with C groups, but Breviscapine could reverse these. As well, glycogen accumulation and injury of glomerular capillary basement membrane were more severe than C groups, but Breviscapine could reverse these changes too. In short, Breviscapine could decrease renal damage from renal pathology.

Metabolic and physiology parameters in experimental mice

As shown in the figure, after 4 weeks of diabetes, both the proteinuria (**Figure 4F**) and serum creatinine (**Figure 4E**) were significantly lower in Breviscapine treatment groups (DB, CIN+B and DCB groups) than D, CIN and DC groups, despite similar levels of body weight (**Figure 4A**), kidney weight (**Figure 4B**) and blood glucose (**Figure 4D**). Furthermore, compared with sham-injected control mice, diabetic mice showed a significant increase in kidney/body weight ratio (**Figure 4C**), although Breviscapine could not reverse it.

Effect of Breviscapine on phosphorylation of PKCβ in CIN in diabetic mice

Given Breviscapine could ameliorate kidney function, decrease ECM expression and Breviscapine was the inhibitor of PKC, we sought to investigate the role of PKCβI and PKCβII in CIN in diabetic mice. We validated that D and DC groups could effectively enhance the activity of phosphorylation of PKCβII but not PKCβI (**Figure 5A**, **5B**). The expression of PKCβII phosphorylation was enriched to about 1.6/1.6-fold in D and DC groups compared with control group. Then we meaningly found Breviscapine could down-regulate 1.2-fold expression of PKCβII phosphorylation in DCB group (**Figure 5A**, **5C**), which was the first time to find that

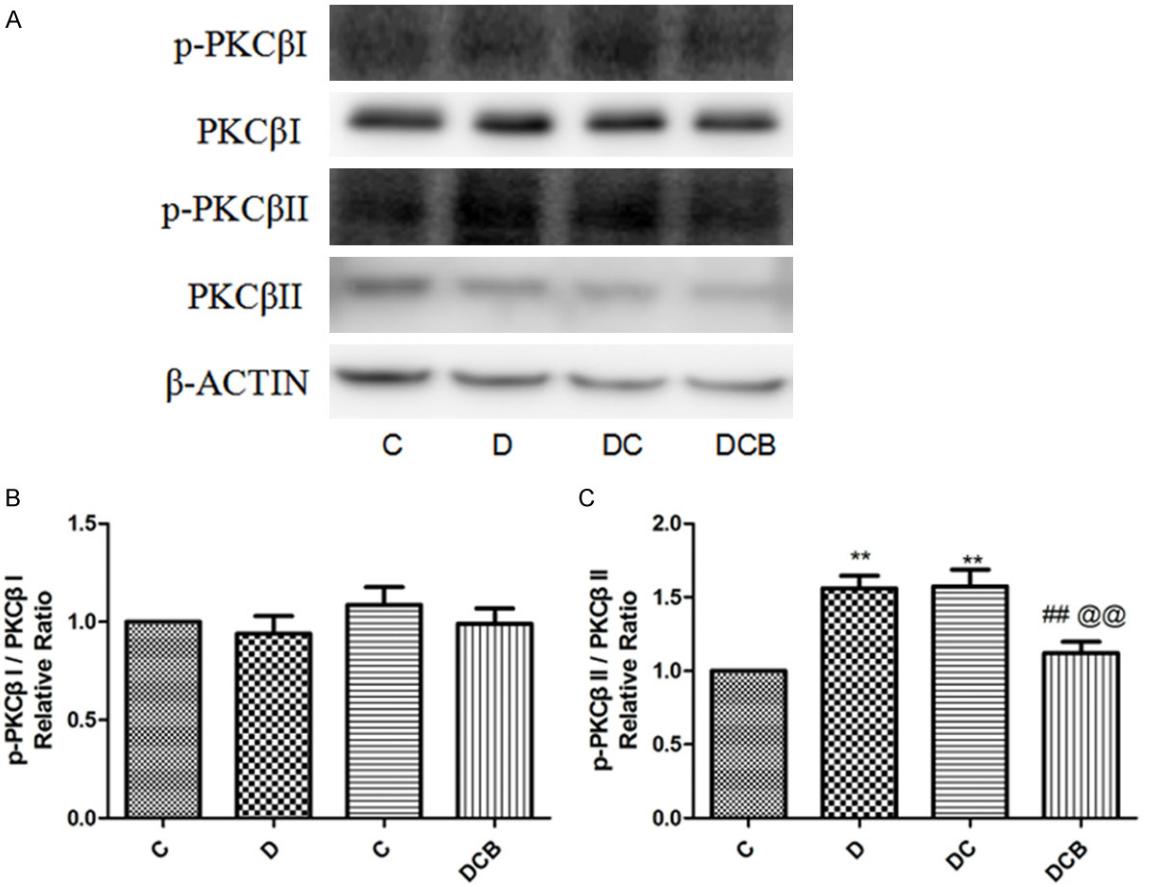

Figure 5. Effect of Breviscapine on phosphorylation of PKCβ in CIN in diabetic mice. A. Western blot of phosphorylation of PKCβI and PKCβII proteins expression in CIN in diabetic mice. B and C. Relative protein expression levels of phosphorylative and total PKCβI and PKCβII are normalised to β-actin and expressed as the mean ± SEM, n = 5. $^{**}p < 0.01$ vs. C group. $^{\#\#}p < 0.01$ vs. D group. $^{@@}p < 0.01$ vs. DC group.

phosphorylation of PKCβII played an important role in the model of CIN in diabetes mellitus.

Breviscapine decreased phosphorylation of Akt as well as MAPK signalling

Breviscapine-mediated products were vital for the function of Akt activity, whereas Akt phosphorylation activity regulated c-Jun N-terminal kinase (c-JNK) 1/2 and p38 (**Figure 6A**). We demonstrated that Akt phosphorylation was 4.7/4.5-fold increase in D/DC groups compared with control mice (**Figure 6B**). There was a 2.1/2.0-fold increase in c-JNK phosphorylation (**Figure 6C**) and 4.4/4.1-fold increase in p38 phosphorylation (**Figure 6D**) in D/DC groups, and significant changes in glycogen synthase kinase-3β (GSK3β) (**Figure 6E**) and extracellular signal-related kinase (ERK1/2) (**Figure 6F**) was not observed. Breviscapine could down-regulate Akt, c-JNK1/2 and p38 phosphorylation expression in DCB groups compared with D and DC groups.

Effect of Breviscapine on the expression of collagen I, CTGF, TGF β1

The present study showed that PKCβII, Akt, c-JNK1/2 and p38 phosphorylation were significantly increased in the D/DC groups compared with these in control mice. At the same time, Breviscapine could reduce the phosphorylation of PKCβII/Akt/c-JNK1/2/p38 in CIN in diabetic mellitus. Thus, we tested the effect of Breviscapine on ECM expression in experimental mice (**Figure 7A**). The protein and RNA expression of collagen I, CTGF, TGF β1 was obviously lower in DCB groups than in DC groups. When compared with DC group, the protein level of collagen I was about 0.4-fold (**Figure 7B**) and the mRNA level of collagen I was at about 0.5-fold (**Figure 7E**) in DCB group; the

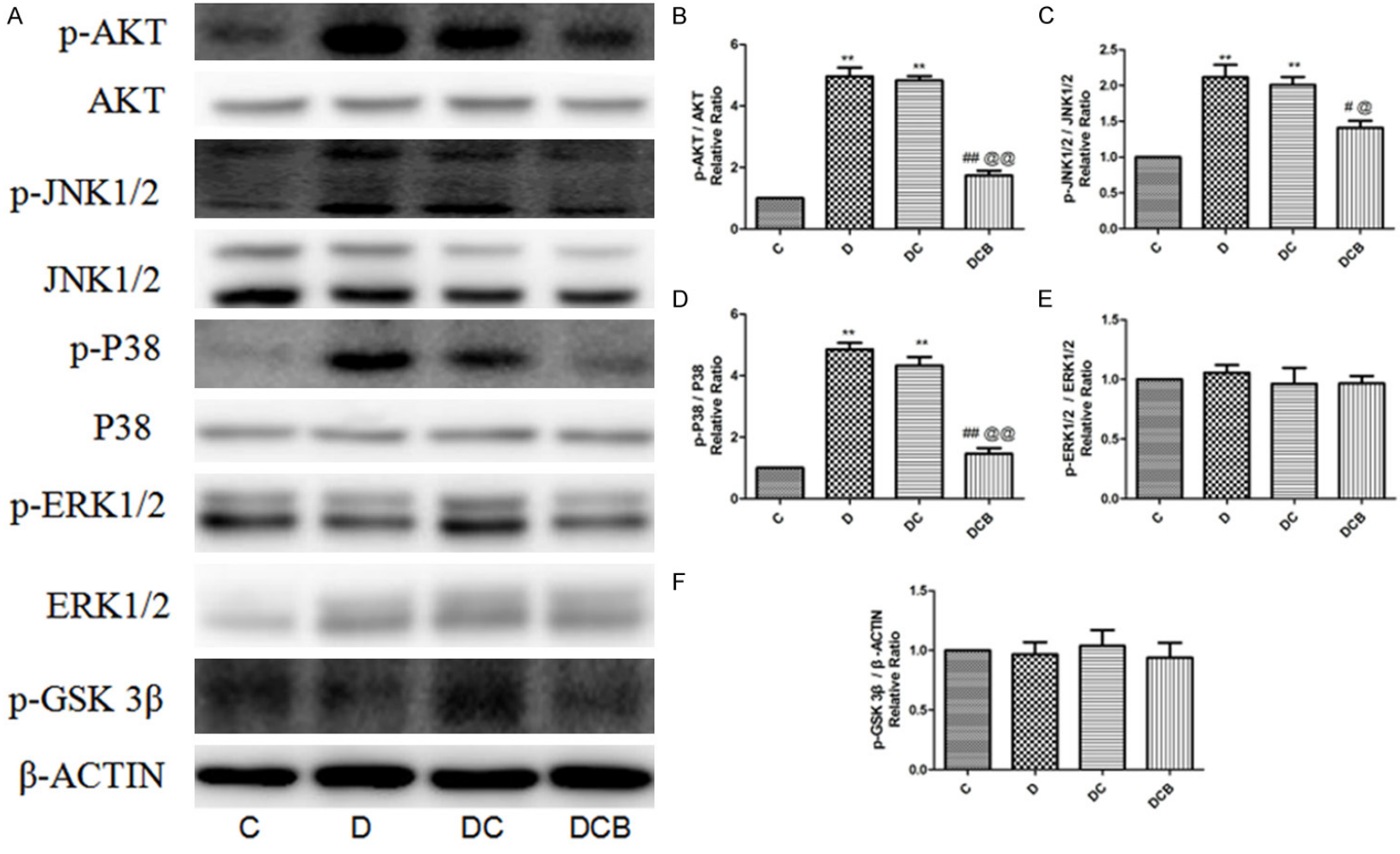

Figure 6. Breviscapine decreased phosphorylation of Akt as well as MAPK signalling. A. Western blot of phosphorylation of Akt and MAPK proteins expression in CIN in diabetic mice. B-F. Relative protein expression levels of phosphorylative and total Akt, JNK1/2, P38, ERK1/2 and GSK3β are normalised to β-actin and expressed as the mean ± SEM, n = 5. $^{**}p < 0.01$ vs. C group. $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$ vs. D group. $^{@@}p < 0.01$ and $^{@}p < 0.05$ vs. DC group.

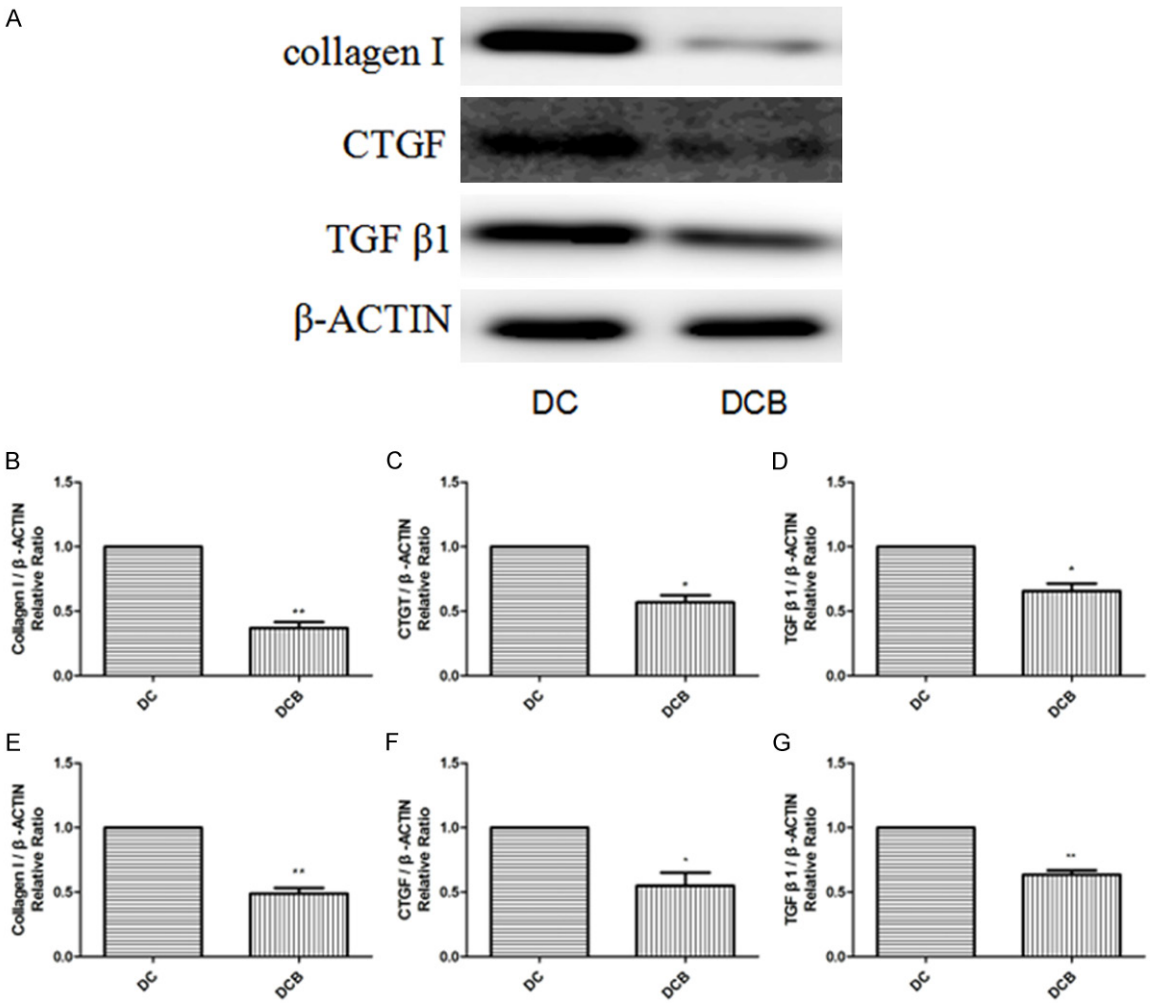

Figure 7. Effect of Breviscapine on the expression of collagen I, CTGF and TGF β1 in CIN of diabetic mice. A. Western blot of collagen I, CTGF and TGF β1 proteins expressions. B-D. Relative protein expression levels of collagen I, CTGF and TGF β1 are normalised to β-actin and expressed as the mean ± SEM, n = 5. $^{**}p < 0.01$ and $^{*}p < 0.05$ vs. DC group. E-G. Relative mRNA expression levels of collagen I, CTGF and TGF β1 are normalised to β-actin and expressed as the mean ± SEM, n = 5. $^{**}p < 0.01$ and $^{*}p < 0.05$ vs. DC group.

protein level of CTGF was about 0.6-fold (**Figure 7C**) and the mRNA level of CTGF was about 0.5-fold (**Figure 7F**) in DCB group; the protein level of TGF β1 was about 0.6-fold (**Figure 7D**) and the mRNA level of TGF β1 was about 0.6-fold (**Figure 7G**) in DCB group. This implied that Breviscapine played a vital role in protecting kidney function via PKCβII/Akt/c-JNK1/2/p38 phosphorylation signalling.

Discussion

In this study we assessed the effect of Breviscapine on the model of CIN via phosphorylation of PKCβII/Akt/JNK1/2/p38 signalling in diabetic mice. We observed that Chinese patent medicine, Breviscapine, significantly reduced renal atrophy, fibrosis, glycogen accumulation, glomerular capillary basement membrane injury, proteinuria, serum creatinine as well as ECM expression in model of CIN in diabetic mellitus. Taken together, these data suggested a role of Breviscapine in the pathology of CIN in diabetic mice.

In recent years, there are more and more researches about reducing the risk of CIN in DM patients [37-39]. Breviscapine had potent anti-renal injury properties [8, 9]. We recently checked that Breviscapine significantly decreased the risk of heart and kidney in diabetic mice [23, 24]. In current study, we found that

there was a significant increase in renal injury biomarkers, including proteinuria and serum creatinine, especially in the mice administrated with CM. Treatment with Breviscapine significantly reversed the effects of CM on serum renal injury markers, as well as proteinuria.

CM induced an increase in the expression of ECM, including collagen I, CTGF, TGF β1 in mice kidney [40]. Breviscapine improved kidney function by decrease the expression of ECM [9]. A body of evidence had demonstrated that Breviscapine had distinctive anti-fibrosis properties, improving kidney injury [8, 9, 16]. In current study, we found that a decrease in products of collagen I, CTGF and TGFβ1. Furthermore, Breviscapine ameliorate renal morphology and fibrosis by H&E and Masson trichrome staining, reduced glycogen and glomerular mesangial matrix accumulation by PAS and PASM staining in our study. The above results suggested the protective effect of Breviscapine not only on diabetic nephropathy but also on CIN in diabetic mice. This may explain why renal injury was less significant in DCB group than in DC group. This was the first time to elucidate the protective effect of Breviscapine in the pathological process.

Although there were a lot of researches about CIN, the mechanism of CIN in diabetic mice was still not completely understood. Most previous studies with specific PKCβ inhibitor ruboxistaurin indicated that PKCβ isoform was primarily responsible for the hyperglycemia-induced renal injury in diabetes mellitus [41-44]. Meier et al. tested this hypothesis by inducing diabetic nephropathy in PKCβ deficient mice and did not find a significant preventive effect of PKCβ deficiency on proteinuria [44]. However, the hyperglycemia-induced renal fibrosis as well as increased expression of ECM was reduced in PKCβ deficiency diabetic mice [45]. Then in our research we used Breviscapine as PKCβ inhibitor as previously described [20-22]. That was consistent with previous studies that PKCβ phosphorylation, especially PKCβII isoform, played an important role, at least partly, in pathogenesis of CIN in diabetic mice. There was a new sight that ECM accumulation in CIN in diabetic mice was induced by PKCβII activation, and Breviscapine could reverse the renal injury. Signalling pathway analysis had demonstrated that diabetes-induced MAPK activation was profibrotic. Therefore, in further elucidating possible mechanisms for the pharmacological response in DC group, we demonstrated that specific down-regulation of Breviscapine attenuated diabetes-induced phosphorylation of JNK1/2 and p38 and its upstream target Akt, which had been implicated in regulating ECM expression.

In conclusion, our results highlighted the importance of Breviscapine in the process of CIN in diabetic mice, which was partly attributed to its suppression of renal fibrosis via inhibiting the phosphorylation of PKCβII/Akt/JNK1/2/p38 signalling pathway.

A limitation of this study is that we could not evaluate the effect of different CM dosage and type on renal injury because of our experimental conditions. Although this study proposed several possible reasons why Breviscapine prevents renal injury, other mechanisms should be explored in future studies. In addition, in-vitro study will need further investigation.

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Disclosure of conflict of interest

None.

Authors' contribution

Fu Guosheng designed the study. Jiang Wen bin, Shen Zhida, He Jialin, Chen Shengyu and Li Zhengwei carried out experiments. Zhang Jiefang, Zhao Wei, Chen Hao, Wu Youyang and Wang Yi analyzed the data. Jiang Wenbin and Li Zhengwei wrote the manuscript. All authors reviewed the manuscript.

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