Mol Biol Rep
DOI 10.1007/s11033-016-3971-5

ORIGINAL ARTICLE

Calcitriol regulates angiotensin-converting enzyme and angiotensin converting-enzyme 2 in diabetic kidney disease

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Received: 3 September 2015 / Accepted: 5 March 2016

Abstract To investigate the effects of calcitriol on angiotensin-converting enzyme (ACE) and ACE2 in diabetic nephropathy. Streptozotocin (STZ) induced diabetic rats were treated with calcitriol for 16 weeks. ACE/ACE2 and mitogen activated protein kinase (MAPK) enzymes were measured in the kidneys of diabetic rats and rat renal tubular epithelial cells exposed to high glucose. Calcitriol reduced proteinuria in diabetic rats without affecting calcium-phosphorus metabolism. ACE and ACE2 levels were significantly elevated in diabetic rats compared to those in control rats. The increase in ACE levels was greater than that of ACE2, leading to an elevated ACE/ACE2 ratio. Calcitriol reduced ACE levels and ACE/ACE2 ratio and increased ACE2 levels in diabetic rats. Similarly, high glucose up-regulated ACE expression in NRK-52E cells, which was blocked by the p38 MAPK inhibitor SB203580, but not the extracellular signal-regulated kinase (ERK) inhibitor FR180204 or the c-Jun N-terminal kinase (JNK) inhibitor SP600125. High glucose down-regulated ACE2 expression, which was blocked by FR180204, but not SB203580 or SP600125. Incubation of cells with calcitriol significantly inhibited p38 MAPK and ERK phosphorylation, but not JNK phosphorylation, and effectively attenuated ACE up-regulation and ACE2 down-regulation in high glucose conditions. The renoprotective effects of calcitriol in diabetic nephropathy were related to the regulation of tubular levels of ACE and ACE2, possibly by p38 MAPK or ERK, but not JNK pathways.

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Keywords Calcitriol · Diabetic kidney disease · Angiotensin converting enzyme · Angiotensin converting enzyme 2

Introduction

The importance of the local renin-angiotensin system (RAS) in diabetic nephropathy has been emphasized [1]. Proximal tubular cells are not only affected secondary to glomerular injury but also are the main sites of damage in hyperglycemia. Chronic exposure of proximal tubular cells to hyperglycemia results in tubulointerstitial damage in diabetic renal disease [2]. Angiotensin II (Ang II) is synthesized in the renal proximal tubular cells, which express all the necessary enzymes and substrates of the entire RAS required for Ang II formation [3]. Angiotensin-converting enzyme (ACE) is a key enzyme that converts angiotensin I (Ang I) into Ang II, thereby causing a sequence of negative pathophysiological effects that aggravate diabetic renal damage. Angiotensin-converting enzyme 2 (ACE2) is a newly discovered RAS family member and a negative regulator of RAS activity. ACE2 is highly expressed in renal tissues and hydrolyzes Ang II to Ang-(1–7), mainly in the kidney proximal tubules [4, 5].

The mitogen activated protein kinase (MAPK) pathway plays a significant role in inflammation, apoptosis and kidney injury in several glomerular and tubulointerstitial diseases, including diabetic kidney disease [6, 7]. At least three MAPK families have been characterized: p38 MAPK, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). Previous studies indicated that activation of all members of the MAPK family were involved in diabetic nephropathy and contributed to the development of diabetic kidney injury [8]. It is widely

Published online: 12 March 2016

accepted that Ang II might activate the MAPK signaling pathway and mediate a series of physiological and pathological responses in diabetic nephropathy. On the other hand, some studies showed that activation of the MAPK cascade signaling networks conversely regulate ACE expression, leading to RAS activation [9].

1α,25-Dihydroxyvitamin D_3 (VD3, the active form of vitamin D) shows a renoprotective action by down-regulating the RAS, with angiotensinogen (AGT) and renin as the main targets [10–12]. The effect of calcitriol on ACE and ACE2 in diabetic nephropathy is poorly understood. The aim of this study was to investigate the impact of calcitriol on ACE/ACE2 and the role of MAPK signaling pathways in diabetic nephropathy.

Methods

Animals and treatment

Wistar rats (Animal Center of HuBei Academy of Medical Sciences, China) weighing 180–220 g were used in our experiments. All rats were treated strictly in accordance with international ethical guidelines. The study was carried out with the approval of the Experimentation Ethics Committee of Wuhan University (Permit Number: 00093380). Animals were randomly divided into four groups: (1) Non-diabetic control rats (vehicle injection), (2) Non-diabetic control rats with calcitriol (vehicle injection and intragastric administration of calcitriol: 0.03 μg/kg per days; Haier Co., China), (3) streptozotocin (STZ)-induced diabetic rats [13] (STZ: 60 mg/kg, ip; Sigma, USA), and (4) STZ-induced diabetic rats with calcitriol (STZ: 60 mg/kg, ip and intragastric administration of calcitriol: 0.03 μg/kg per days). The diabetic state was assessed in STZ-treated rats by detecting the blood glucose levels after 72 h. Only rats with blood glucose concentration higher than 300 mg/dL were selected for this study.

At the end of 16 weeks, blood and 24-h urine samples were collected for detection of urinary β_2-microglobulin (β_2-MG), urinary albumin (Alb), urinary creatinine (UCr), serum calcium (Ca), serum phosphorus (P) and serum intact-parathyroid hormone (iPTH) (performed by the Zhongnan Hospital of Wuhan University Biochemistry Laboratory, Wuhan, China). Kidneys were immediately removed for further analysis. Renal ACE and ACE2 enzymatic activity was assessed using a fluorometric assay as previously described [14, 15]. Data are presented in fluorescence units per minute normalized to the total protein concentration.

Immunohistochemistry

The antigen was exposed by treatment with boiling citrate buffer (0.01 M, pH 6.0). Samples were incubated with primary monoclonal antibody against ACE (1:100; Epitomics, USA) and ACE2 (1:200; Abcam, USA) overnight at 4 °C. They were then incubated with the ChemMate™ EnVision™/HRP kit (Gene Company Ltd.; Hong Kong, China). Color was developed with the diaminobenzidine (DAB) substrate and slides were mounted. The areas of ACE and ACE2 staining (cytoplasm) were identified in each digitized image from five sections of each kidney ($n = 5$/group). The data are reported using Image J software version 1.42q.

Cell culture and treatment

Rat renal tubular epithelial cells (NRK-52E; ATCC, catalogue # CRL-1571) are immortalized cells. The cells are passage 29 when received and passage 30–31 when used in our study. They were grown at 37 °C in a humidified atmosphere of 5 % CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA), supplemented with 10 % fetal bovine serum. Cells were randomly divided into seven groups: (1) normal glucose (5.5 mmol/L); (2) high glucose (30 mmol/L); (3) normal glucose with calcitriol; (4) high glucose with calcitriol; (5) high glucose with FR180204 (ERK1/2 inhibitor; Calbiochem, USA); (6) high glucose with SB203580 (p38 MAPK inhibitor; Calbiochem); and (7) high glucose with SP600125 (JNK inhibitor; Calbiochem). NRK-52E cells were preincubated for 2 h with inhibitors (FR180204, SB203580, SP600125) or 12 h with calcitriol, and subsequently stimulated with high glucose for 24 h. Each treatment was repeated in triplicate for the following assays.

Western blot analysis

Homogenized kidney tissue and cells were lysed in lysis buffer. Protein concentration was measured by the Bradford method [16]. Proteins were loaded on a SDS-PAGE and transferred to nitrocellulose membranes. The blots were incubated with primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies (1:1000; Pierce, USA). Band densities were quantified using the Scion Image software. The primary antibodies used were anti-p38 MAPK, anti-ERK1/2 and anti-JNK (1:1000; Cell Signaling, USA), anti-ACE, anti-ACE2 and β-actin (1:500; Santa Cruz, USA).

Real-time PCR

Total RNA was isolated using the TRIzol system (Invitrogen Life Technologies, USA). cDNA synthesis was performed using the First Strand cDNA Synthesis Kit (Toyobo, Japan). Real-time PCR was performed with the THUNDERBIRD SYBR qPCR Mix (Toyobo) with the

following parameters: 95 °C for 1 min, 95 °C for 30 s, and 58 °C for 20 s, 72 °C for 25 s, and 72 °C for 5 min with steps 2, 3 and 4 repeated for 35 cycles. β-actin was used as a control. RT-PCR was performed with the following oligonucleotide primers: ACE [AGA TGT TTT GGC TCT GTC TGT GT (sense) and TGG TTG TAG TTC TCC TTG GTG AT (antisense)], ACE2 [GGC AGA CGT ATG GGT GAG TG (sense) and GAC AGG AGG CTC GTA AGG TG (antisense)] and β-actin [CGT TGA CAT CCG TAA AGA CCT (sense) and TAG GAG CCA GGG CAG TAA TCT (antisense)].

Statistical analysis

All results are presented as mean ± SD. Statistical differences were assessed using analysis of variance (ANOVA) and Student–Newman–Keuls (SNK)-q test. $P<0.05$ indicates statistical significance.

Results

Calcitriol reduces urinary β_2-MG and Alb/UCr in STZ-induced diabetic rats without affecting calcium-phosphorus metabolism

Urinary β_2-MG and urinary Alb/UCr in diabetic rats were higher than values in control rats. Calcitriol reduced urinary β_2-MG and Alb/UCr by 30.6 and 38.4 %, respectively, in diabetic rats. Calcitriol did not affect urinary β_2-MG or Alb/UCr in non-diabetic control rats (Fig. 1). There were no statistical differences in CCr, serum Ca, P or iPTH among different groups (Table 1).

Calcitriol regulates ACE/ACE2 and MAPK phosphorylation in STZ-induced diabetic rats

Immunohistochemical staining showed that ACE and ACE2 expression was strong in tubules but weak in glomeruli of both the control and diabetic groups (Fig. 2). Semi-quantitative analysis of immunohistochemical staining and Western blot showed increased ACE and ACE2 levels in diabetic rats (Figs. 2, 4a). The increase in ACE level was more than that of ACE2, leading to an elevated ACE/ACE2 ratio (Figs. 2c, 4a). As illustrated in Fig. 3, both ACE and ACE2 activity increased in kidneys of STZ-induced diabetic rats, but the increase in ACE activity was more than that of ACE2. Calcitriol treatment down-regulated ACE and up-regulated ACE2 expression and activity. Calcitriol did not affect ACE and ACE2 in non-diabetic control rats (Figs 2, 3, 4a).

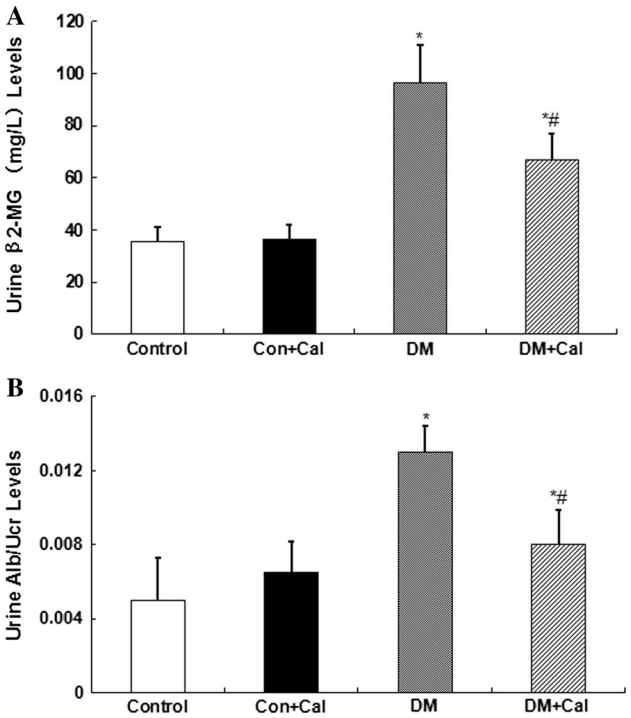

Fig. 1 Calcitriol reduced urinary β_2-MG and urinary Alb/UCr in STZ-induced diabetic rats. Control group (control); control group with calcitriol (Con + Cal); diabetic group (DM); diabetic group with calcitriol (DM + Cal). Results are presented as the mean ± SD. $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ veruss DM

Western blots showed p38 MAPK, ERK and JNK phosphorylation was greater in diabetic rats compared to that in control rats. Calcitriol treatment attenuated p38 MAPK and ERK phosphorylation, but not that of JNK. Expression levels of total p38 MAPK, ERK and JNK proteins were unchanged (Fig. 4).

Calcitriol regulates high glucose-induced MAPK activation in NRK-52E cells

High glucose increased p38 MAPK phosphorylation (1.7-fold), ERK phosphorylation (1.4-fold) and JNK phosphorylation (1.5-fold) in NRK-52E cells. Calcitriol attenuated the high glucose-induced phosphorylation of p38 MAPK and ERK, but not that of JNK. In contrast, expression of total p38 MAPK, ERK and JNK proteins was unchanged (Fig. 5). Calcitriol did not affect MAPK phosphorylation in normal glucose conditions (data not shown).

Calcitriol regulates high glucose-induced ACE/ACE2 levels in NRK-52E cells

High glucose up-regulated ACE expression in NRK-52E cells, and this effect was blocked by SB203580 (p38 MAPK inhibitor), but not FR180204 (ERK1/2 inhibitor) or

Table 1 Effect of calcitriol on blood glucose, Ccr, serum Ca, P and iPTH in STZ-induced diabetic rats

Parameter	Control	Con + Cal	DM	DM + Cal
Body weight (g)	261 ± 14	259 ± 17	211 ± 18*	223 ± 18*
Blood glucose (mg/dL)	98.0 ± 10.8	102.0 ± 11.2	420 ± 92*	410 ± 79*
Ccr (mL/min)	1.63 ± 0.18	1.83 ± 0.19	1.98 ± 0.22	1.87 ± 0.27
Ca (mmol/L)	2.34 ± 0.003	2.31 ± 0.002	2.35 ± 0.003	2.32 ± 0.002
P (mmol/L)	2.12 ± 0.01	2.22 ± 0.02	2.24 ± 0.01	2.20 ± 0.02
iPTH (ng/L)	8.13 ± 0.04	8.09 ± 0.03	7.93 ± 0.01	8.11 ± 0.02

Results are expressed as the mean ± SD (n = 10 per group). Control group (control); control group with calcitriol (Con + Cal); diabetic group (DM); diabetic group with calcitriol (DM + Cal). *$P < 0.05$ versus control

SP600125 (JNK inhibitor). Conversely, high glucose down-regulated ACE2 expression in NRK-52E cells, and this effect was blocked by FR180204, but not SB203580 or SP600125. Calcitriol attenuated the high glucose-induced down-regulation of ACE level and up-regulation of ACE2 level in NRK-52E cells (Fig. 6). Calcitriol did not affect expression of either ACE or ACE2 in normal glucose conditions (data not shown).

Discussion

RAS activity plays a key role in diabetic kidney injury [17]. Research shows that kidney tubular cells express all the RAS components, including ACE and ACE2 [18]. ACE is a key component of the RAS, and its enzymatic product Ang II is the primary effector of the RAS and plays a key role in the pathogenesis of diabetic nephropathy. Activation of the renal ACE/Ang II/AT1 axis results in harmful effects, including hypertension, inflammation, oxidative stress and renal fibrosis. However, recent studies indicate that ACE2/angiotensin-(1–7) [Ang-(1–7)]/Mas receptor axis also play a key role in diabetic nephropathy.

ACE2 is a recently discovered homologue of ACE. It shares 40–42 % homology with ACE but possesses different biochemical roles. ACE2 converts Ang II into Ang-(1–7), which exerts protective effects against the generation of reactive oxidative species, apoptosis, renal fibrosis and inflammation by interactions with the Mas receptor [19]. Therefore, the ACE2/Ang-(1–7)/Mas axis has been suggested to act as a negative regulator with effects opposite to those of ACE/AngII/AT1. The balance between intrarenal ACE and ACE2 activities may, therefore, determine Ang II levels [20–23]. A few studies have shown that low, high and unchanged ACE2 expression were observed in diabetic rats [24–28]. The differences in results of these studies regarding renal ACE and ACE2 expression may be attributed to racial differences and analytical methods or, more likely, disease duration, the degree of severity of diabetic kidney damage, or structural differences in the renal cortex or whole kidney tissue samples.

Our study demonstrated that ACE2 expression increased in STZ-induced diabetic rats. Interestingly, this was not seen in the cell culture model. High glucose reduced ACE2 levels in NRK-52E cells. We speculate that the discrepancy may be due to the complicated physiological environment in vivo that is not fully replicated in the in vitro model. In vivo, increased ACE2 expression might be a compensatory effect to down-regulate Ang II accumulation in the early stage of diabetic kidney disease to adapt to overactivity of Ang II. This hypothesis, however, needs to be verified in future experiments.

Furthermore, our study also showed that compared with control rats, both tubular ACE and ACE2 expression in diabetic rats significantly increased. The increase in ACE levels was higher than that of ACE2 levels, resulting in an elevated ACE/ACE2 ratio, which indicates that in the etiology of diabetic kidney injury, ACE2 and ACE may both be activated in vivo. Although they are antagonistic, the imbalance between ACE and ACE2 may alter the balance in RAS, resulting in the accumulation of Ang II and eventually in kidney tissue injury.

Vitamin D receptor knockout mice exhibit more severe nephropathy in the obstructed kidney compared with wild-type mice [29]. A possible mechanism is related to its anti-inflammatory activity [30]. Many experiments have indicated that vitamin D may be a potent immunomodulatory agent [31, 32]. Recently, active vitamin D was shown to be a protective factor for microvascular complications (principally diabetic retinopathy and nephropathy) and macrovascular complications in people with diabetes [33, 34]. Vitamin D deficiency is highly prevalent in diabetic subjects. The prevalence of vitamin D deficiency was 70.8 % in subjects with diabetes-related chronic kidney disease (CKD), 38.8 % in subjects with non-diabetic CKD and 41 % in subjects with diabetes without advanced CKD [35].

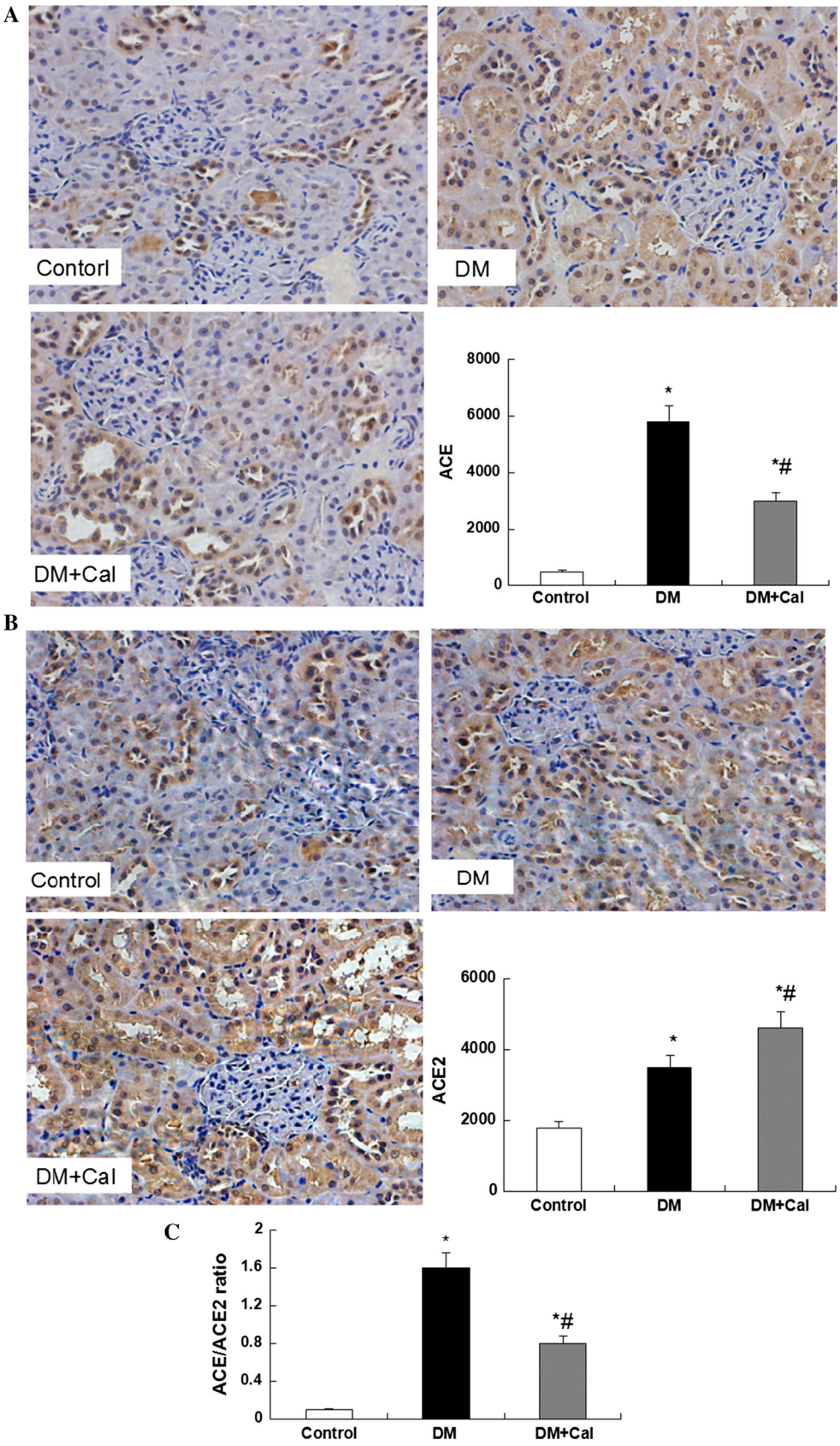

Fig. 2 **a** Calcitriol regulated tubular ACE expression in STZ-induced diabetic rats (Immunohistochemical staining × 200). Control group (control); diabetic group (DM); calcitriol treatment group (DM + Cal). Results are presented as the mean ± SD. $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus DM. **b** Calcitriol regulated tubular ACE2 expression in STZ-induced diabetic rats (Immunohistochemical staining × 200). Control group (control); diabetic group (DM); calcitriol treatment group (DM + Cal). Results are presented as the mean ± SD. $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus DM. **c** Calcitriol regulated tubular ACE/ACE2 ratio in STZ-induced diabetic rats (Immunohistochemical staining semi-quantitative analysis). Control group (control); Diabetic group (DM); calcitriol treatment group (DM + Cal). Results are presented as the mean ± SD. $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus DM

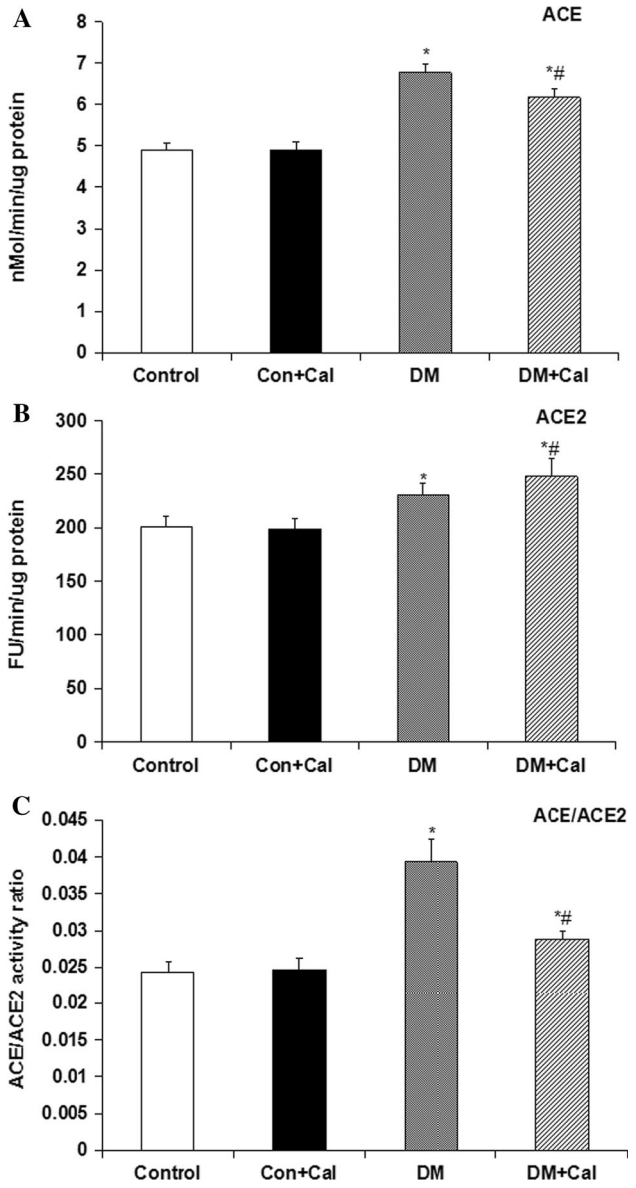

Fig. 3 Calcitriol regulated ACE/ACE2 activity in STZ-induced diabetic rats. Control group (control); control group with calcitriol (Con + Cal); diabetic group (DM); diabetic group with calcitriol (DM + Cal). Results are presented as the mean ± SD. $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus DM

A recent randomized controlled trial (VITAL study) reported that paricalcitol inhibited the RAS and is a novel approach to decrease residual renal risk in patients with diabetic nephropathy [36]. In this experiment, we also observed that calcitriol reduced proteinuria without affecting calcium or phosphorus metabolism in diabetic rats. Although the renoprotective effect of calcitriol has recently been well documented [37–40], the underlying mechanism remains unclear. There is increasing evidence that calcitriol is a negative endocrine regulator of the RAS. Research has shown that calcitriol decreases renin biosynthesis and thereby suppresses RAS activity [8]. Renal AGT levels in VDR knockout mice were significantly higher than those in wild-type mice. A vitamin D analog inhibits AGT expression in diabetic mice [41]. In the diabetic state, inhibitors of both VDR and RAS blocked the development of renal damage when given alone, but the combination (VDR-agonist + RAS-inhibitor) synergistically blunted the development of diabetic kidney disease by suppressing intrarenal RAS [42].

In the current experiments, we found that calcitriol up-regulates tubular ACE expression and down-regulates ACE2 expression both in vivo and in vitro. This result indicates that calcitriol is not only a potent endocrine suppressor of renin and AGT biosynthesis, but also a regulator of ACE and ACE2 expression. However, further studies are needed to explore the precise mechanism.

High-glucose incubation or hyperglycemia markedly stimulates the phosphorylation of MAPKs, which may mediate macrophage infiltration, inflammation and kidney dysfunction [6]. Inhibition of MAPK signaling effectively avoided high glucose-induced kidney damage, suggesting that MAPK may be a new therapeutic target for diabetic nephropathy. However, the exact relationship between the MAPK pathway and the RAS in the pathophysiology of diabetic kidney disease is still unclear. Our results showed the importance of the MAPK signaling pathway in the modulation of ACE and ACE2 expression in high glucose-incubated renal cells. This finding indicated that high glucose up-regulated ACE level in NRK-52E cells, and this effect was blocked by SB203580 (p38 MAPK inhibitor), but not FR180204 (ERK1/2 inhibitor) or SP600125 (JNK inhibitor). Meanwhile, high glucose down-regulated ACE2 expression, and this effect was blocked by FR180204, but not SB203580 or SP600125. Incubation of cells with calcitriol significantly inhibited phosphorylation of p38 MAPK and ERK, but not that of JNK induced by high glucose. Further, calcitriol attenuated the high glucose-induced up-regulation of ACE and down-regulation of ACE2 in a dose-dependent manner.

As illustrated in Fig. 7, the results described above suggest that calcitriol regulates ACE and ACE2 in NRK-52E cells in high-glucose conditions possibly via ERK or p38 MAPK signaling pathways. However, the molecular mechanism by which p38 MAPK and ERK modulate ACE and ACE2 is still unclear. Further experiments are required to determine transcriptional mechanisms involved in these pathways.

In conclusion, the current findings suggest that the renoprotective actions of calcitriol may be related to the regulation of tubular ACE and ACE2 expression, especially the ACE/ACE2 ratio, via p38 MAPK and/or ERK signaling pathways in diabetic nephropathy. Therefore, our

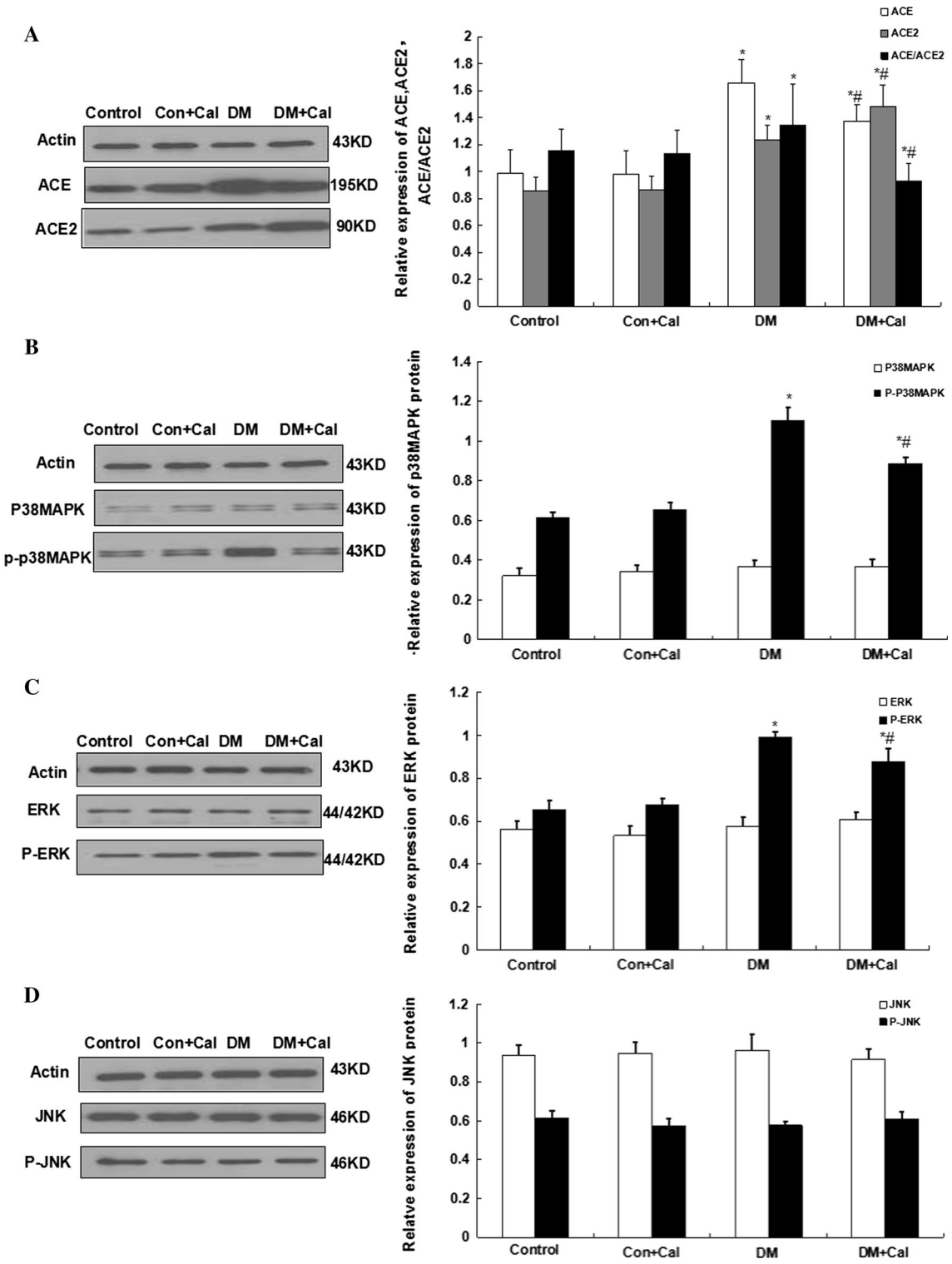

Fig. 4 Calcitriol regulated ACE/ACE2 expression and MAPK phosphorylation in STZ-induced diabetic rats. Control group (control); control group with calcitriol (Con + Cal); diabetic group (DM); diabetic group with calcitriol (DM + Cal). Results are presented as the mean ± SD. $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus DM

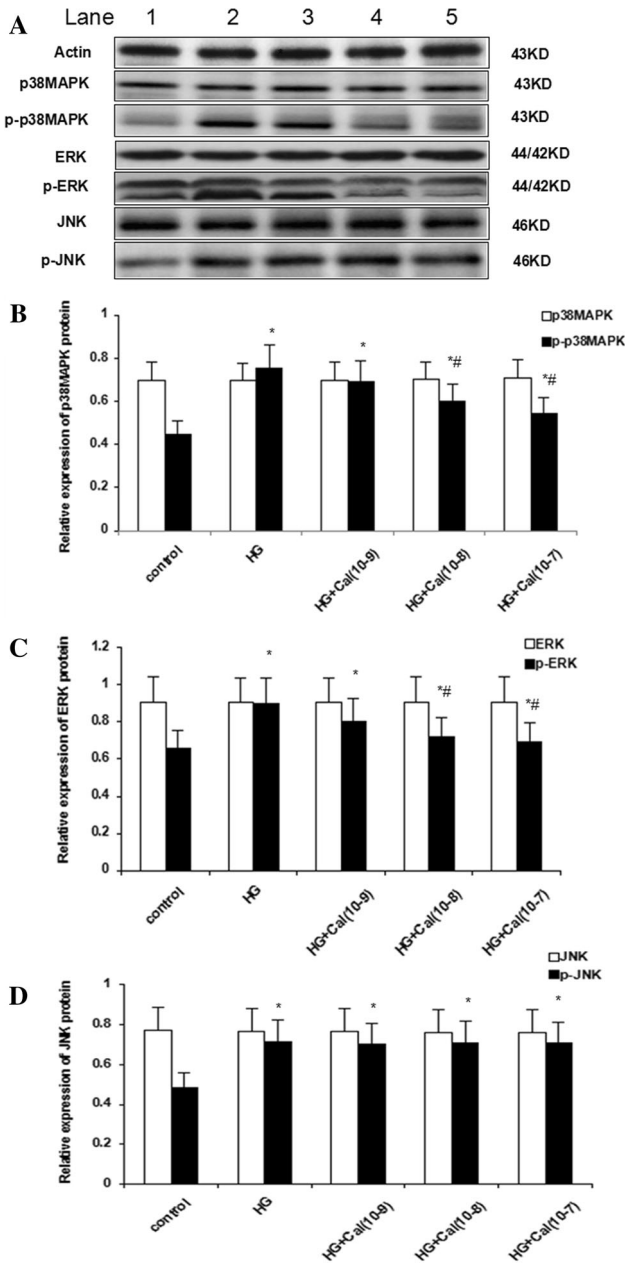

Fig. 5 Calcitriol regulated MAPK phosphorylation induced by high glucose in NRK-52E cells. **a** Western blot of phospho-p38 MAPK, phosphor-ERK, phosphor-JNK and total p38 MAPK, ERK, JNK; **b** Semi-quantitative analysis of p38 MAPK protein; **c** Semi-quantitative analysis of ERK protein; **d** Semi-quantitative analysis of JNK protein. *Lane 1* Control [normal glucose (5.5 mmol/L)]; *lane 2* HG [high glucose (30 mmol/L)]; *lane 3* HG + Cal 10^{-9} [high glucose + calcitriol (10^{-9} mol/L)]; *lane 4* HG + Cal 10^{-8} [high glucose + calcitriol (10^{-8} mol/L)]; and *lane 5* HG + Cal 10^{-7} [high glucose + calcitriol (10^{-7} mol/L)]. Results are expressed as the mean ± SD. Results are presented as the mean ± SD. $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus high glucose

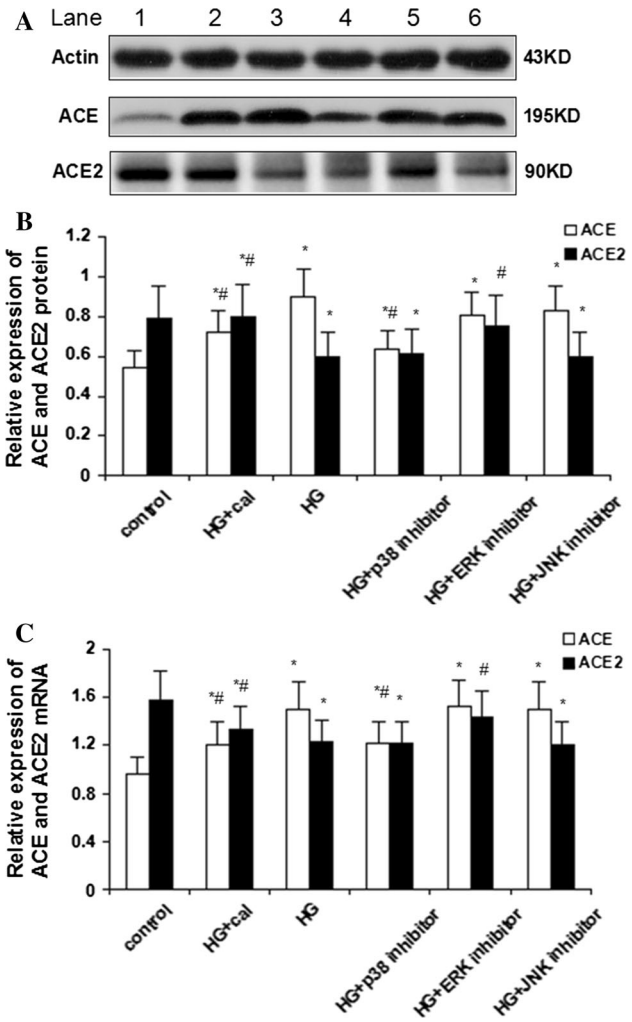

Fig. 6 Calcitriol regulated high glucose-induced ACE/ACE2 levels in NRK-52E cells. **a** Western blot of ACE and ACE2 protein expression. *Lane 1* control [normal glucose (5.5 mmol/L)]; *lane 2* HG + Cal [high glucose (30 mmol/L) + calcitriol (10^{-7} mol/L)]; *lane 3* HG [high glucose (30 mmol/L)]; *lane 4* HG + p38 MAPK inhibitor [high glucose + SB203580 (10 μM)]; *lane 5* HG + ERK1/2 inhibitor [high glucose + FR180204 (10 μM)]; and *lane 6* HG + JNK inhibitor (high glucose + SP600125 10 μM). **b** Semi-quantitative analysis of ACE/ACE2 protein expression. **c** qRT-PCR of ACE and ACE2 mRNA expression. Results are expressed as the mean ± SD. Results are presented as the mean ± SD. $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus high glucose

work provides important and new insights into the mechanism of action of calcitriol. The regulation of RAS in the renoprotection mechanism has significant therapeutic implications for diabetic nephropathy.

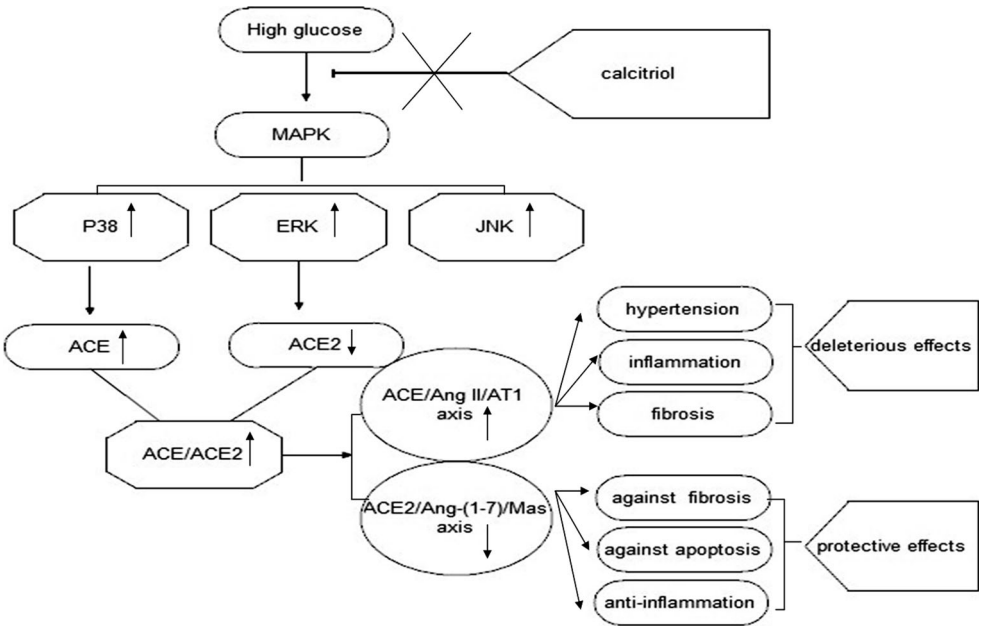

Fig. 7 Probable mechanism of by which calcitriol regulates ACE and ACE2 in diabetic nephropathy

Funding This study was supported by the National Natural Science Foundation of China (No. 81000304) and Hubei Provincial Natural Science Foundation (No. 2010CDB00402).

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