Inhibitory effect of tumor necrosis factor-α on the basolateral Kir4.1/Kir5.1 channels in the thick ascending limb during diabetes

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Abstract. Diabetic nephropathy is a major contributor to the morbidity and mortality of patients with diabetes. TNF-α expression is elevated during diabetes and is implicated in the pathogenesis of diabetic nephropathy; however, its underlying molecular mechanisms remain unclear. The present study aimed to investigate the effect and molecular mechanism of TNF-α on the basolateral inwardly rectifying potassium (Kir)4.1/Kir5.1 channels in the thick ascending limb (TAL) of rat kidneys using western blotting and the patch clamp technique to provide a theoretical basis for the cause of the decrease in kidney concentrating capacity during diabetes. The results demonstrated that urinary TNF-α excretion and protein TNF-α expression in the TAL increased and basolateral Kir4.1/Kir5.1 channel activity decreased during diabetes; however, diabetic rats exhibited amelioration of Kir4.1/Kir5.1 activity with a soluble TNF-α antagonist, TNF receptor fusion protein (TNFR:Fc). These results suggested that TNF-α inhibited the activity of the basolateral Kir4.1/Kir5.1 channel in the TAL of rat kidneys during diabetes. In addition, the protein expression levels of phospholipase A_2 (PLA_2) and cyclooxygenase-$_2$ (COX_2) increased in diabetic rats, the effects of which deceased following treatment with TNFR:Fc compared with the diabetic group. Furthermore, an agonist of PLA_2 (melittin) and COX_2 production [prostaglandin E_2 (PGE_2)] inhibited the basolateral Kir4.1/Kir5.1 channels. Taken together, the results of the present study suggested that the inhibitory effect of TNF-α on the basolateral Kir4.1/Kir5.1 channels in the TAL during diabetes is mediated by the $PLA_2/COX_2/PGE_2$ signaling pathway.

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Introduction

Diabetes mellitus is a group of metabolic diseases characterized by chronic hyperglycemia from defective insulin secretion and/or impaired biological effects (1). Diabetic nephropathy (DN) is a major microvascular complication of diabetes mellitus and the leading cause of end-stage renal disease, thereby contributing to the high mortality rates (2).

Several mechanisms contribute to the onset and development of DN, including hemodynamic factors, oxidative stress and cytokine signaling (3,4). Recently, increasing evidence has suggested that inflammation plays a key role in the pathogenesis of DN, although it is commonly considered a non-inflammatory disease (5,6). TNF-α, a potent proinflammatory cytokine, is synthesized and released by infiltrating macrophages and intrinsic kidney cells (7). Previous studies have reported that increased urinary TNF-α, as a pathogenic factor, may precede the appearance of pathological albuminuria, and thus is considered a marker of kidney injury in early stages of DN (8,9). Several alterations in renal function occur during the initial stages of DN, including a decrease in urinary concentrating ability; however, its molecular mechanism remains unclear.

The thick ascending limb (TAL) of the Henle's loop is responsible for the reabsorption of 20-25% of filtered NaCl, which is the most important step required to establish the hyperosmotic gradient of the medulla for the concentration of urine (10,11). The basolateral K^+ channels in the TAL play a critical role in sustaining the transepithelial membrane transport by generating the cell membrane potential to drive Cl^- diffusion (12,13). Previous studies have demonstrated that the basolateral Kir4.1/Kir5.1 heterotetramers, with a conductance of 40-50 pS, are the predominant subtype in the TAL, and indirectly influence the tubular NaCl transportation by regulating the activity of Na^+-K^+-$2Cl^-$ cotransporters (NKCC2), which further affects urinary concentrating (14,15). The main effects of impaired urinary concentrating ability in the kidney include renal polyuria, or even renal diabetes insipidus, and infection with the loss of immune substances in the urine.

The present study aimed to investigate the effect of TNF-α on the basolateral Kir4.1/Kir5.1 channels in the TAL during diabetes. Furthermore, the study sought to determine its underlying regulatory mechanism to provide a theoretical basis for the detection of impaired kidney concentrating capacity during diabetes.

Materials and methods

Reagents. Antibodies against TNF-α (ab6671), phospholipase A_2 (PLA_2, AF6329), COX_2 (AF7003), Kir4.1 (DF9260) and Kir5.1 (K009361P) were purchased from Abcam, Affinity and Solarbio. Melittin, prostaglandin E_2 (PGE_2), polylysine and collagenase were purchased from Sigma-Aldrich; Merck KGaA. TNF receptor fusion protein (TNFR:Fc) was purchased from CPGJ Pharmaceutical Co., Ltd. (http://27919267.b2b.11467.com/). The TNF-α (900TM73) and PGE_2 ELISA kits (EK7124) were purchased from PeproTech, Inc., and Boster Biological Technology, respectively. The rat albumin ELISA kit (ab23564) was purchased from Abcam.

Animals and experimental design. Male pathogen-free Sprague-Dawley rats (weight, 200±20 g, 6-7 weeks old) were obtained from the Animal Facility of Jiamusi University, and housed at 20-25˚C, 50-65% relative humidity and with a 12-h light/dark cycle, with free access to normal food and water. A total of 40 rats were randomly divided into four groups: Control rats, diabetic rats, control rats treated with TNFR:Fc (control + TNFR:Fc) and diabetic rats treated with TNFR:Fc (diabetic + TNFR:Fc). Diabetic rats were induced via intraperitoneal injection of 60 mg/kg streptozotocin (STZ) dissolved in citric acid buffer. The levels of fasting blood glucose were monitored by drawing blood from the tail vein 72 h after STZ injection. The rats with fasting blood glucose >16.7 mmol/l and increased drinking water, eating and urine volume were considered to be successful diabetic models. Subcutaneous injection of TNFR:Fc (2 mg/kg) was performed twice a week in the control and diabetic rats before STZ injection for 3 weeks (16). All animal experiments were approved by the Medical Ethics Committee of Jiamusi University (Jiamusi, China; approval no. JMSU-229).

Measurement of urine output and urinary albumin. Rats were placed in metabolic cages to collect urine from 9 am to 9 am the next day, and the supernatant of urine following centrifugation was the 24 h urine output. Urinary albumin (UAlb) was measured using the ELISA kit for rat albumin, according to the manufacturer's instructions.

Measurement of TNF-α in urine and PGE_2 in tissues. The levels of TNF-α in urine and PGE_2 in tissues were measured using the TNF-α rat ELISA and PGE_2 rat ELISA kits, respectively, according to the manufacturer's instructions. Briefly, the standard solution and samples were added to the wells and the plates were incubated at 37˚C for 90 min. After washing three times with washing buffer, the plates were incubated with corresponding antibody working liquid for 60 min at room temperature. Subsequently, the plates were re-washed and incubated with ABC working solution at 37˚C for 30 min. Following addition of the TMB substrate, the plates were incubated for 10 min at 37˚C in the dark. Absorbance was measured at a wavelength of 450 nm, using a spectrophotometer (BioTek Instruments, Inc.), after adding the stop solution. The levels of TNF-α and PGE_2 were quantified according to the standard curve.

Preparation of the TAL tissues. Rats were anesthetized using pentobarbital (50 mg/kg) and sacrificed via cervical dislocation. The kidneys were immediately removed and cut into 1-mm-thick sections after removing the capsule and poles. The renal cortex and inner stripe of outer medulla were carefully excised and minced with a blade under a dissecting microscope. Samples were incubated and shaken in HEPES buffer solution containing 10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$ and 1.8 mM $CaCl_2$ (pH 7.4), with collagenase type 1A (1 mg/ml) at 37˚C for 5 min. Undigested tissues were subjected to three treatments with collagenase (5 min each) and the supernatants were combined. The combined supernatants were subsequently filtered through 180 and 50 μm nylon mesh membranes, and the TALs retained on the 50 μm mesh were collected for western blotting.

Western blotting. Protein samples (30 μg) were extracted from the TAL tissues using RIPA lysis buffer, separated via 10 or 12% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with blocking solution containing 5% non-fat milk in TBS-0.05% Tween (TBS-T) for 1 h at room temperature and subsequently incubated with the corresponding primary antibody at 4˚C for 12 h. Membranes were washed four times with TBS-T (15 min each) and subsequently incubated with the secondary antibody (ZB-2301; OriGene Technologies, Inc.) solution containing 5% non-fat dry milk in TBS-T for 1 h at room temperature, prior to re-washing with TBS-T (4x15 min). Protein bands were visualized using ECL plus chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.) and analyzed using ImageJ software (version 1.45s; National Institutes of Health).

Patch clamp technique. The 1-mm-thick sections were incubated in HEPES buffer with collagenase type 1A at 37˚C for 40-60 min. The digested TALs were isolated under a dissecting microscope and placed on a cover glass (5x5 mm) coated with polylysine. The cover glass with TALs was transferred to a chamber filled with HEPES-buffered NaCl solution (in mM: 140 NaCl, 5 KCl, 1.5 $MgCl_2$, 1.8 $CaCl_2$ and 10 HEPES, pH 7.4) and mounted on an inverted microscope (Nikon Corporation). Using a P-97 electrode-puller, the patch clamp electrodes were filled with a pipette solution (in mM: 10 HEPES, 140 KCl and 1.8 $MgCl_2$, pH 7.4) and fixed to the probe to patch the treated TALs. The channel currents were low-pass filtered at 0.5 kHz and recorded using an Axon 700B patch clamp amplifier. The data were digitized with an Axon interface (Digidata 1400A) and analyzed using pClamp 10.0 software (Axon Instruments; Molecular Devices, LLC). The channel activity, expressed as a product of channel number and open probability (*NPo*), was calculated from data samples of 90 sec durations at a steady state, as follows: $NPo=\Sigma (1t_1+2t_2+...it_i)$, in which t_i is the fractional open time spent at each of the observed current levels.

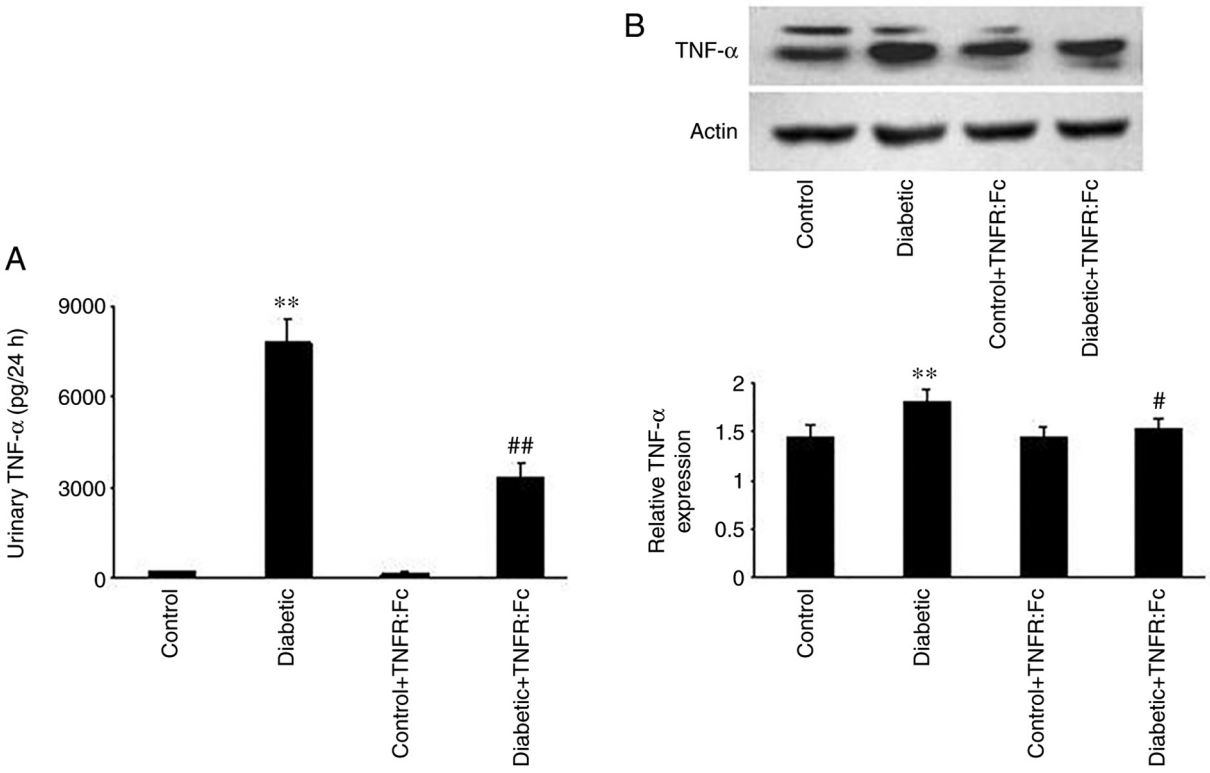

Figure 1. Changes in urinary TNF-α excretion and TNF-α protein expression in the TAL during diabetes. (A) Urinary TNF-α excretion. (B) TNF-α protein expression in the TAL. **P<0.01 vs. control group; #P<0.05 and ##P<0.01 vs. diabetic group. TAL, thick ascending limb; TNFR:Fc, TNF receptor fusion protein.

Melittin treatment. After patching and recording the current of the K^+ channel for 2-3 min, melittin (5 μM) was added and the current of the K^+ channel was recorded for 3-5 min.

Statistical analysis. Statistical analysis was performed using SPSS software version 19.0 (IBM Corp.). Data were presented as the mean ± standard error of the mean and analyzed using a one-way ANOVA followed by a Tukey's post hoc test and Student-Newman-Keuls post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Changes in urinary TNF-α excretion and TNF-α protein expression in the TAL during diabetes. To observe the changes in TNF-α levels during diabetes, urinary TNF-α excretion and TNF-α protein expression in the TAL were measured. The results demonstrated that urinary TNF-α excretion in diabetic rats was markedly increased compared with the control rats, and decreased following treatment with TNFR:Fc in the diabetic + TNFR:Fc group (n=7; P<0.01; Fig. 1A). Furthermore, relative TNF-α expression (Fig. 1B) was significantly higher in the diabetic group compared with the control group (n=5; P<0.01), while treatment with TNFR:Fc decreased its level in diabetic + TNFR:Fc rats (n=5; P<0.05). These results confirm that TNF-α expression is upregulated during diabetes.

Changes in blood glucose, UAlb and urine output during diabetes. Compared with the control rats, the levels of blood glucose, UAlb and urine output in diabetic rats were significantly increased (n=7; P<0.01; Table I). Compared with the diabetic rats, UAlb levels in the diabetic + TNFR:Fc group were markedly decreased (n=7; P<0.01; Table I), while no significant changes were observed in blood glucose and urine output levels following treatment with TNFR:Fc in the diabetic + TNFR:Fc group.

Effect of TNF-α on the basolateral Kir4.1/Kir5.1 channels in the TAL during diabetes. Increasing evidence has suggested that basolateral Kir4.1/Kir5.1 channels in the TAL play an important role in determining NKCC2 activity and influencing tubular NaCl transportation and urine concentration (10). Thus, Kir4.1/Kir5.1 protein expression was detected in each group via western blotting to investigate the effect of TNF-α on the basolateral K^+ channel in the TAL during diabetes. As presented in Fig. 2, relative Kir4.1/Kir5.1 protein expression was significantly decreased in diabetic rats compared with normal rats (n=5; P<0.01), and increased in the diabetic + TNFR:Fc group following treatment with TNFR:Fc (n=5; P<0.01). Taken together, these results suggest that TNF-α inhibits the activity of basolateral Kir4.1/Kir5.1 in the TAL during diabetes.

Role of the PLA_2-dependent pathway in the inhibitory effect of TNF-α on Kir4.1/Kir5.1 during diabetes. After revealing the inhibitory effect of TNF-α on Kir4.1/Kir5.1 during diabetes, the present study aimed to determine its underlying molecular mechanism. Previous studies have reported that the activity of the basolateral K^+ channel is often mediated by the PLA_2-dependent pathway (12). Thus, the role of the PLA_2-dependent pathway in the inhibitory effect of TNF-α on Kir4.1/Kir5.1 during diabetes was investigated. As presented

Table I. Changes in blood glucose, UAlb and urine output during diabetes.

Group	Blood glucose, mmol/1	UAlb, mg/24 h	Urine output, ml/24 h
Control	5.31±0.45	0.29±0.04	9.12±1.33
Diabetic	25.47±3.09[a]	1.17±0.25[a]	166.75±13.40[a]
Control + TNFR:Fc	5.39±0.82	0.31±0.06	8.96±1.28
Diabetic + TNFR:Fc	25.39±2.98	0.58±0.07[b]	143.25±21.82

[a]P<0.01 vs. control group; [b]P<0.01 vs. diabetic group. Data are presented as the mean ± standard error of the mean. UAlb, urinary albumin; TNFR:Fc, TNF receptor fusion protein.

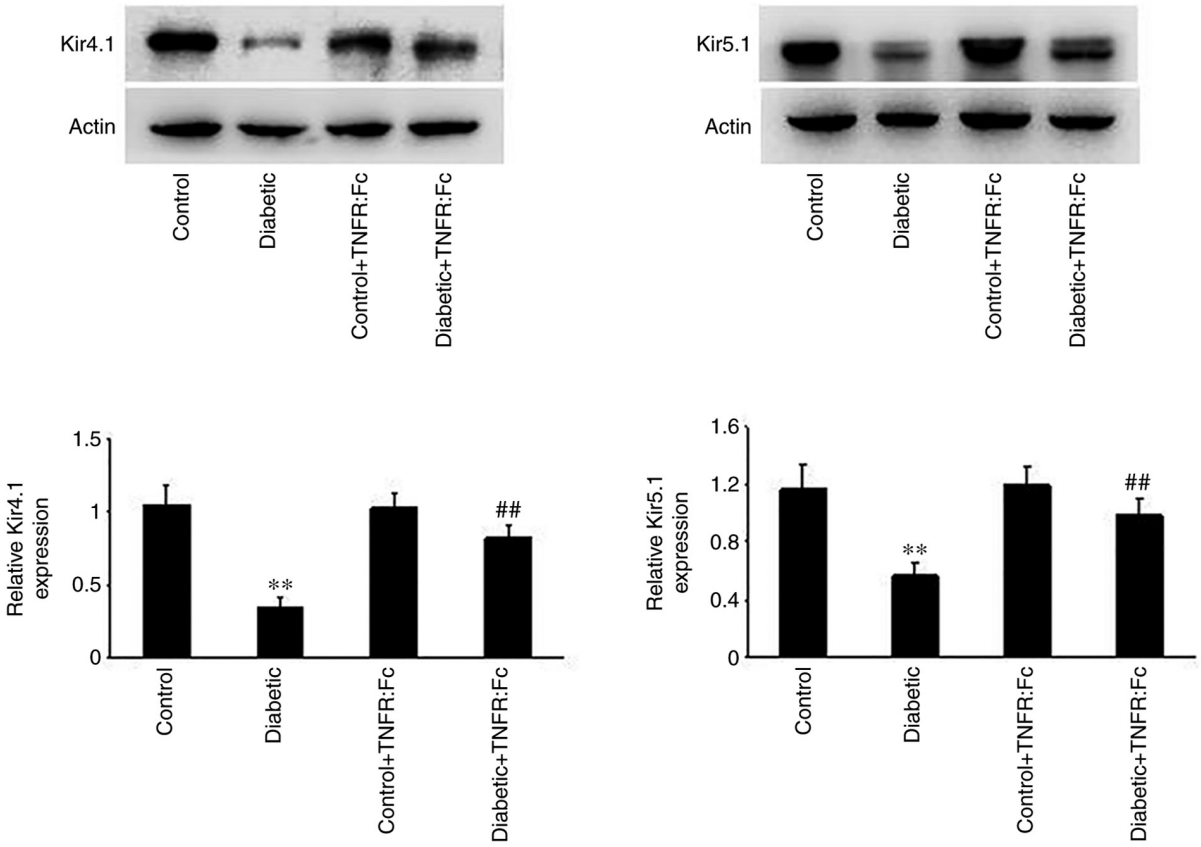

Figure 2. Effect of TNF-α on the basolateral Kir4.1/Kir5.1 channels in the thick ascending limb during diabetes. **P<0.01 vs. control group; ##P<0.01 vs. diabetic group. TNFR:Fc, TNF receptor fusion protein; Kir, inwardly rectifying potassium channels.

in Fig. 3, relative PLA_2 expression was significantly higher in diabetic rats compared with normal rats (n=5; P<0.01), but decreased in the diabetic + TNFR:Fc group compared with diabetic rats following treatment with TNFR:Fc (n=5; P<0.05). Thus, the PLA_2-dependent pathway may participate in the regulatory effect of TNF-α on Kir4.1/Kir5.1 during diabetes. To further determine the role of the PLA_2-dependent pathway, the effect of melittin, an agonist of PLA_2 (17), on Kir4.1/Kir5.1 in the TAL was investigated via the patch clamp technique. The results demonstrated that addition of melittin (5 μM) decreased the channel activity (NP_o) from 0.33±0.07 to 0.08±0.02 in a cell-attached patch (n=5; P<0.01, Fig. 4). Collectively, these results suggest that the inhibitory effect of TNF-α on Kir4.1/Kir5.1 during diabetes is mediated by the PLA_2-dependent pathway.

Role of the cyclooxygenase-$_2$ (COX_2)/PGE_2 pathway in the inhibitory effect of TNF-α on Kir4.1/Kir5.1 during diabetes. As a downstream pathway of the PLA_2-dependent pathway, the COX_2/PGE_2 pathway is associated with the development of diabetes (18). However, whether it is involved in the regulation of TNF-α on Kir4.1/Kir5.1 during diabetes remains unclear. Thus, the role of the COX_2/PGE_2 pathway in the inhibitory effect of TNF-α on Kir4.1/Kir5.1 during diabetes was also investigated in the present study. Western blot analysis was performed to detect COX_2 protein expression in the TAL. As presented in Fig. 5A, relative COX_2 protein expression was significantly higher in diabetic rats compared with normal rats (n=5; P<0.01), but decreased in the diabetic + TNFR:Fc group compared with diabetic rats following treatment with TNFR:Fc (n=5; P<0.05). Subsequently, PGE_2 expression was

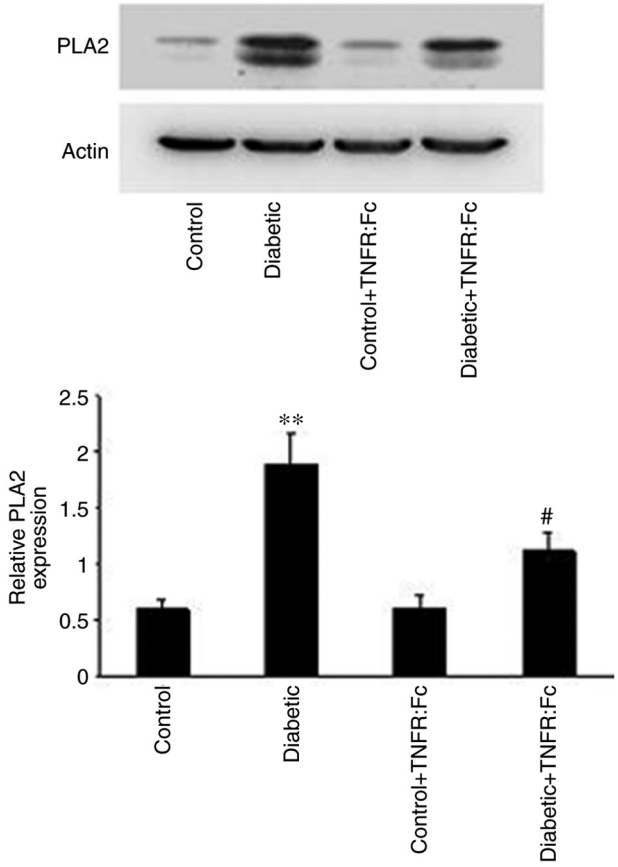

Figure 3. Change in protein expression of PLA_2 in the thick ascending limb during diabetes. **P<0.01 vs. control group; #P<0.05 vs. diabetic group. TNFR:Fc, TNF receptor fusion protein; PLA_2, phospholipase A_2.

measured in the TAL via ELISA. As presented in Fig. 5B, PGE_2 expression was markedly increased in diabetic rats compared with normal rats, while treatment with TNFR:Fc decreased PGE_2 expression in the diabetic + TNFR:Fc group (n=5; P<0.01). The patch clamp technique was performed to determine the effect of PGE_2 on Kir4.1/Kir5.1 during diabetes. The results demonstrated that the NP_o decreased from 0.31±0.06 to 0.09±0.03 following treatment with 10 μM PGE_2 (n=5; P<0.01; Fig. 6). Taken together, these results suggest that the COX_2/PGE_2 pathway is involved in the inhibitory effect of TNF-α on Kir4.1/Kir5.1 during diabetes.

Discussion

The present study aimed to investigate the effect of TNF-α, elevated by diabetes, on the Kir4.1/Kir5.1 in the TAL. The results demonstrated that TNF-α inhibited the activity of Kir4.1/Kir5.1 via the $PLA_2/COX/PGE_2$ pathway during diabetes. Currently, three lines of evidence support this concept. First, TNF-α expression in the TAL was significantly increased in diabetic rats, and decreased following treatment with TNFR:Fc. Secondly, the protein expression levels of PLA_2 and COX_2 were higher in the TAL of diabetic rats, and were decreased following treatment with TNFR:Fc. Thirdly, addition of melittin or PGE_2 inhibited the channel activity of Kir4.1/Kir5.1 in a cell-attached patch. Thus, it was hypothesized that increased TNF-α expression during diabetes activates the $PLA_2/COX_2/PGE_2$ pathway, thereby inhibiting basolateral Kir4.1/Kir5.1 channel activity in the TAL.

The Na^+ and Cl^- load filtered from the glomerulus is reabsorbed in the TAL via two steps: First, they enter epithelial cells via apical NKCC2 and then, they leave the cells via basolateral Na^-K^+ pump and Cl^- channels, respectively (19). It is well-known that basolateral K^+ channels play an important role in the modulation of NaCl transportation by affecting cell membrane potential under physiological conditions (20). Activation of basolateral K^+ channels increases the negativity of the cell membrane potential, thereby augmenting the driving force for Cl^- exit, while inhibition of basolateral K^+ channel activity depolarizes the cell membrane potential, thereby diminishing the driving force for the diffusion of Cl^- across the basolateral membrane (21). Consequently, inhibition of Cl^- diffusion leads to an increase in intracellular Cl^- concentration, which suppresses the interaction between WNK lysine deficient protein kinase 3 and serine/threonine kinase 39, and inhibits NKCC2 activity by decreasing the phosphorylation of NKCC2 (22). Given that the active reabsorption of NaCl in the water-impermeable TAL is essential for the urinary concentrating mechanism, inhibition of NaCl reabsorption in the TAL under pathological conditions decreases the urinary concentrating ability (13). It has been reported that diabetic nephropathy impairs urinary concentrating ability (23).

DN is considered a form of 'microinflammation', whereby several cytokines are involved in its underlying immunopathological mechanisms (24). Among these, TNF-α is an important mediator of inflammatory tissue damage and a major participant in the pathogenesis of DN (25). Consistent with experimental models, clinical investigations have reported that serum and urinary concentrations of TNF-α in diabetic patients with nephropathy are higher than non-diabetic subjects or diabetic patients without renal involvement (16). Enhanced TNF-α is cytotoxic to renal cells and can cause direct renal injury by promoting inflammation and the accumulation of extracellular matrix, decreasing glomerular blood flow, inducing apoptosis and damaging glomerular permeability barrier (26,27). TNF-α can also indirectly disrupt the barrier function of the glomerular capillary wall and enhance the albumin permeability by inducing the production of reactive oxygen species in diverse cells, including mesangial cells (28). In addition, Battula *et al* (29) demonstrated that increased TNF-α production in response to hypercalcemia inhibits NKCC2 activity and NaCl reabsorption via the COX_2/PGE_2 pathway, which contributes to polyuria and concentration defects.

There is a distinct association between PLA_2 and the COX-PG system (30). PLA_2 enzymes are the upstream regulators of liberating free arachidonic acid from the sn-2 position of membrane phospholipids (31,32). Arachidonic acid is released from phospholipids via the action of PLA_2 and converted into PGs via COXs (33). PGE_2 is a prominent prostanoid produced in the kidney, which is involved in diverse renal functions regulating hemodynamics and tubular salt and water transport (34). COXs, including $COX\text{-}_1$ and $COX\text{-}_2$, are rate-limiting enzymes in the PGE_2 synthesis pathway (35). While no major renal pathology has been reported for $COX\text{-}_1$ knockout mice, $COX\text{-}_2$-lacking mice display abnormalities in renal development and severe nephropathy (36,37). Previous studies have reported that renal $COX\text{-}_2$ activity and PGE_2 production are

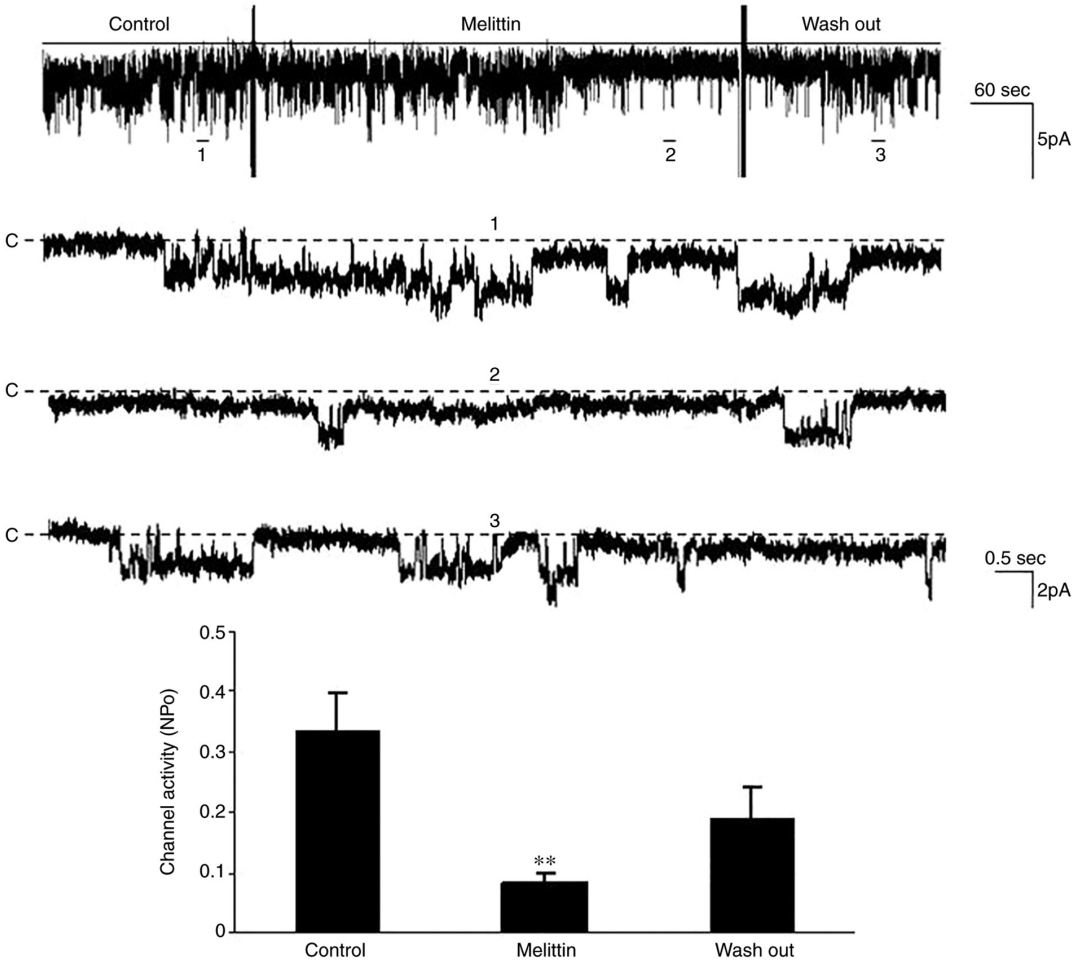

Figure 4. Effect of 5 μM melittin on the Kir4.1/Kir5.1 channel activity. The experiment was recorded using a cell-attached patch clamp. The top trace represents the experimental time course. The other traces with numbers represent the fast time resolution. The holding potential was 0 mV, and the channel closed current is indicated by 'C'. **P<0.01 vs. control group. NPo, channel number and open probability; Kir, inwardly rectifying potassium channels.

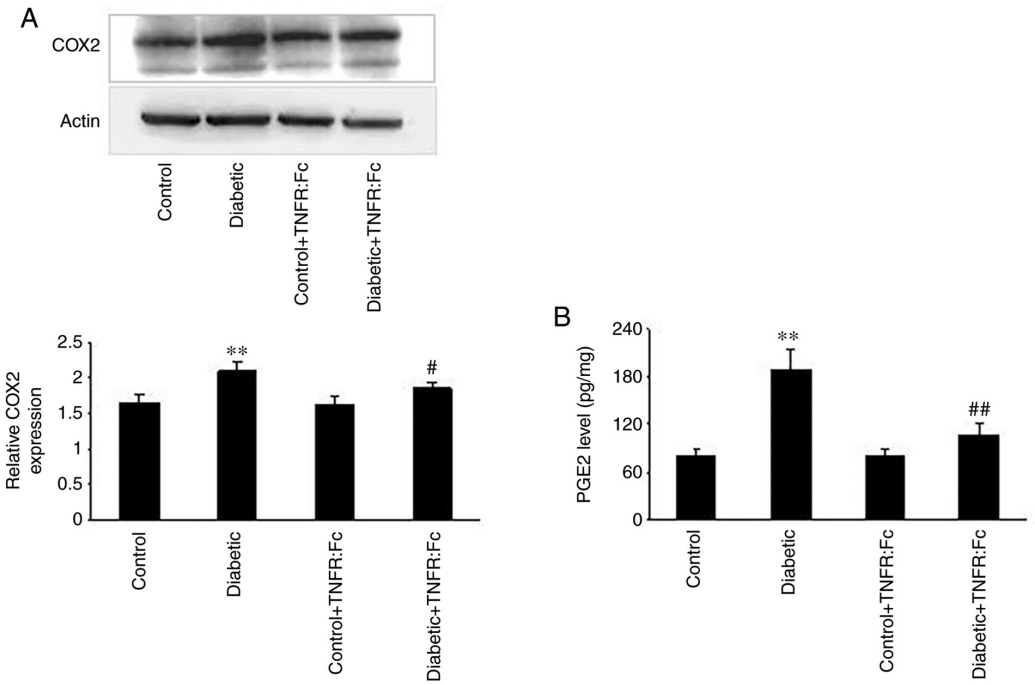

Figure 5. Changes in protein expression of COX_2 and PGE_2 level in the TAL during diabetes. (A) Protein expression of COX_2 in the TAL. (B) PGE_2 level in the TAL. **P<0.01 vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. diabetic group. COX_2, cyclooxygenase-$_2$; PGE_2, prostaglandin E_2; TAL, thick ascending limb; TNFR:Fc, TNF receptor fusion protein.

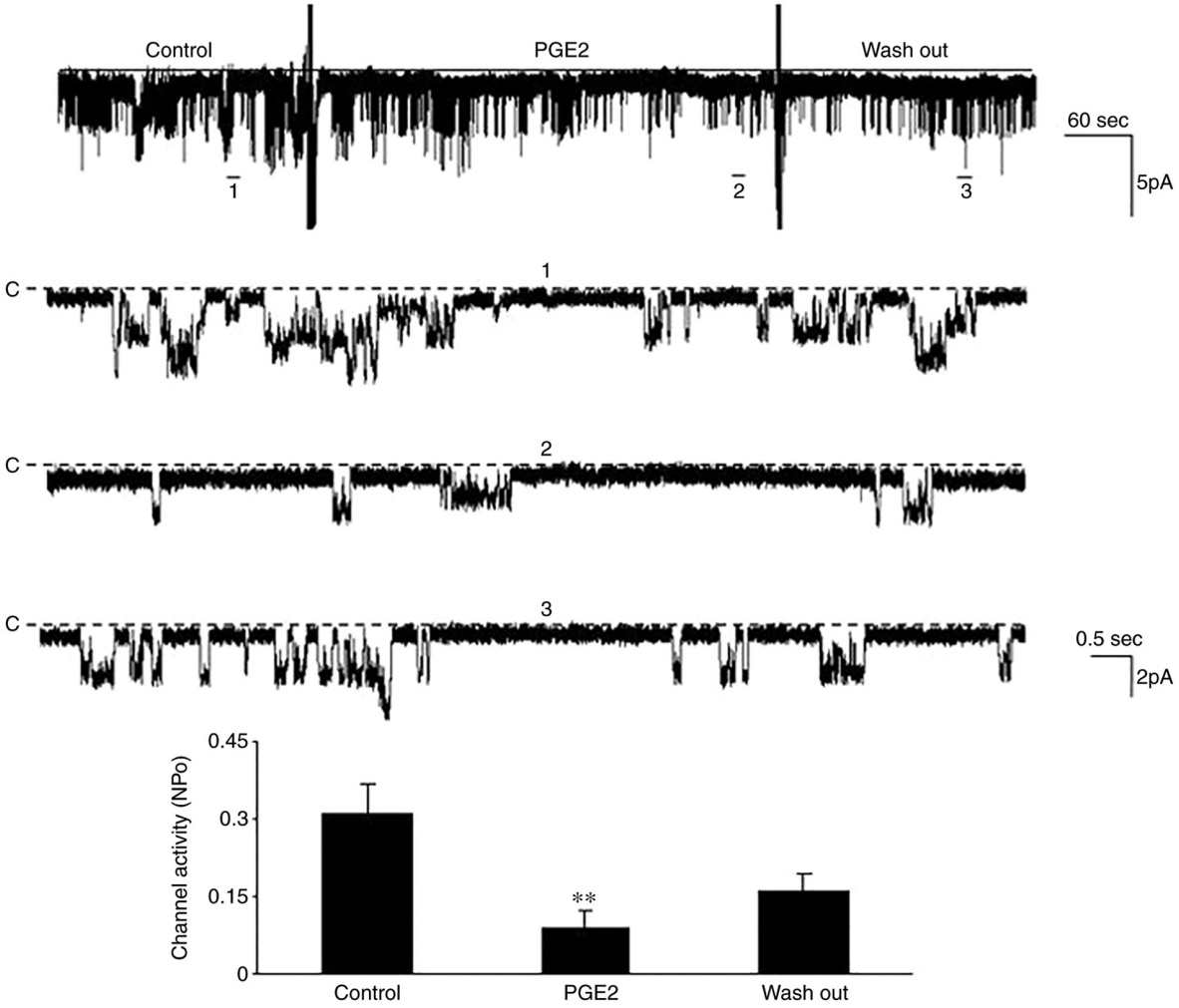

Figure 6. Effect of 10 μM PGE_2 on the Kir4.1/Kir5.1 channel activity. The experiment was recorded by a cell-attached patch clamp. The top trace represents the experimental time course. The other traces with numbers represent the fast time resolution. The holding potential was 0 mV, and the channel closed current is indicated by 'C'. **P<0.01 vs. control group. NPo, channel number and open probability; PGE_2, prostaglandin E_2; Kir, inwardly rectifying potassium channels.

elevated in diabetes mellitus, which contributes to the pathogenesis of DN (38). Inhibition of COX_2 has been demonstrated to reverse some of the renal complications of STZ-diabetes, such as attenuating glomerulosclerosis and glomerular hypertrophy, thereby slowing the development of proteinuria (36,39).

The results of the present study and previous findings suggest that the COX_2/PGE_2 pathway is involved in the pathogenesis of diabetic nephropathy and the impairment of urinary concentrating ability; however, its underlying molecular mechanisms remain unclear. The findings of the current study demonstrated that the inhibitory effect of TNF-α on the basolateral Kir4.1/Kir5.1 channels in the TAL during diabetes occurred via regulation of the $PLA_2/COX_2/PGE_2$ pathway. Given that the basolateral K^+ channels determine the driving force for Cl^- diffusion across the basolateral membrane (23), a TNF-α-induced decrease in channel activity of Kir4.1/Kir5.1 during diabetes may be associated with a decrease in NaCl reabsorption in the TAL and urine concentration. Thus, the results presented in the present study provide a novel mechanism by which TNF-α impairs urinary concentrating ability during diabetes, which occurs via stimulation of the $PLA_2/COX_2/PGE_2$ pathway to inhibit the activity of the basolateral Kir4.1/Kir5.1 channels in the TAL.

Only some pathological changes in early stage of diabetic nephropathy were observed in the present study, others in middle or late stages of diabetic nephropathy will be done in the future study in order to comprehensively explore the pathogenesis of diabetes and find effective prevention and treatment methods.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

GZ and ZL performed the experiments. YZ and RC acquired and analyzed data. XZ and WW analyzed data and drafted the manuscript. HS designed the project and revised the manuscript. All authors have read and approved the final manuscript. GZ and HS confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of Jiamusi University (Jiamusi, China; approval no. JMSU-229).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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