

Mycophenolic acid inhibits the phosphorylation of nuclear factor- κ B and Akt in renal tubular epithelial cells

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Abstract. Renal tubulointerstitial injury induced by albumin overload is a critical stage during the progression of renal interstitial fibrosis and progression of chronic renal diseases. Inosine-5'-monophosphate dehydrogenase inhibitor mycophenolate mofetil (MMF), a pro-drug of mycophenolic acid (MPA), is known to attenuate the progression of renal interstitial fibrosis; however, the underlying molecular mechanisms of the anti-fibrotic effects of derivatives of MMF have not yet been studied. The present study assessed the effects of the MPA on renal tubular epithelial cells. Transforming growth factor beta 1 (TGF- β_1) has been indicated to have a central role in the underlying molecular mechanisms of renal fibrosis; furthermore, nuclear transcription factor- κ B (NF- κ B) is a transcription factor associated with the production of inflammatory cytokines, cell proliferation and apoptosis. In addition, the Akt signaling pathway has important roles in cell proliferation, differentiation, metabolism and apoptosis. The present study subjected the NRK52E rat kidney epithelial-derived cell line to albumin overload, which resulted in an increase in TGF- β_1 production as well as phosphorylation of Akt and the binding activity of NF- κ B to the promoter region of the TGF- β_1 gene, which was, however, reduced following pre-incubation of the cells with MPA. In addition, the effects of albumin were partially blocked by Ly294002, a specific inhibitor of Akt. In conclusion, the results of the present study suggested that MPA may exert its anti-fibrotic effects by inhibiting the upregulation of TGF- β_1 and the activation of NF- κ B following albumin overload, which may be partly dependent on the Akt pathway.

Introduction

The process of chronic renal diseases is typically accompanied with progressive renal fibrosis and the inhibition of fibrogenesis appears to be an attractive therapeutic target. Re-absorption of albumin by renal epithelial cells is important in the progression of renal tubular fibrosis caused by proteinuria (1). Mycophenolate mofetil (MMF), a pro-drug of mycophenolic acid (MPA), is one of the most frequently used immunosuppressive drugs for the prophylaxis of allograft rejection after renal, cardiac or liver transplantation. It is known that MPA is a potent, selective, non-competitive and reversible inhibitor of inosine-5'-monophosphate dehydrogenase. MPA inhibits not only the proliferation of lymphocytes, but also that of other mesenchymal cells (2-4). Transforming growth factor beta 1 (TGF- β_1) has a central role in fibrosis. Following combination of TGF- β_1 with its receptor, numerous signaling pathways are activated, including the Smad signaling pathway and the phosphoinositide-3 kinase (PI3K)/Akt pathway (5-7). Nuclear factor- κ B (NF- κ B) is a transcription factor associated with the production of inflammatory factors, cell proliferation and apoptosis, which is involved in numerous processes of inflammatory signal transduction. Activation of the transcription factor NF- κ B is known to drive renal inflammation and fibrosis (8). Akt, a member of the serine/threonine protein kinase superfamily and the PI3K/Akt signaling pathway, has important roles in cell proliferation, differentiation, metabolism and apoptosis (9).

In spite of previous evidence of albumin-induced expression of TGF- β_1 and NF- κ B, this mechanism has remained to be demonstrated in renal epithelial cells (10). The present study assessed the effects of MPA on NRK52E rat renal epithelial cell line in order to test the hypothesis that MPA inhibits albumin-induced expression of TGF- β_1 and activation of NF- κ B in renal epithelial cells through the Akt pathway.

Materials and methods

Chemicals and reagents. The NRK52E normal rat kidney epithelial-derived cell line was obtained from the American Type Culture Collection (CRL-1571; Manassas, VA, USA). Dulbecco's modified Eagle's medium, nutrient mixture F-12 (DMEM/F12; 1:1), fetal bovine serum (FBS) and trypsin/EDTA solution were purchased from GE Healthcare (Little Chalfont, UK). TRIzol reagent were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA).

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Primers were obtained from Sangon Biological Engineering Technology and Services (Shanghai, China). Rabbit anti-rat phosphorylated (p)-Akt monoclonal antibody (cat. no. 4060) and rabbit anti-rat β -actin monoclonal antibody (cat. no. 4970) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Nuclear proteins were extracted using the N-Extract kit (Sigma-Aldrich, St. Louis, MO, USA) and total protein concentration was determined using the Bio-Rad detergent-compatible protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Akt inhibitor Ly294002 (9) and MPA were purchased from Sigma-Aldrich.

Cell culture and reagents. NRK52E cells were maintained in monolayer culture in 75 cm² Falcon T-flasks (Thermo Fisher Scientific) containing DMEM/F-12 supplemented with 4% fetal calf serum, 15 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 20 mmol/l sodium bicarbonate, 0.5 mmol/l sodium pyruvate, 17.5 mmol/l glucose, streptomycin and penicillin (Invitrogen; Thermo Fisher Scientific) at 37°C in an incubator with a humidified atmosphere with 5% CO₂ in air. The cells were grown to 40% confluence, washed with serum and sodium pyruvate-free DMEM/F-12, and subsequently incubated with MPA or Ly294002. Untreated cells served as a control.

Experimental groups and treatments. NRK52E cells were divided into the following groups: Control group (untreated); albumin group (treated with 30 mg/ml albumin; Sigma-Aldrich); Ly294002 group (treated with 10 μ mol/l Ly294002 for 30 min and then with 30 mg/ml albumin); and the MPA group (30 mg/ml albumin + 10 μ mol/l MPA). Following incubation for 12 h, cells were harvested for the subsequent assays.

Reverse-transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from NRK52E cells using TRIzol following the manufacturer's instructions. Subsequently, 2 μ g total RNA was reverse-transcribed with avian myoblastosis virus reverse transcriptase (Promega Corporation, Madison, WI, USA). PCR amplification was then performed; in brief, 50 pmol/l PCR primers for β -actin and TGF- β ₁ were added to each reaction mixture containing 0.2 mmol/l deoxynucleoside triphosphates (Promega Corporation), 3 mmol/l MgSO₄ and 1 U DNA polymerase (Promega Corporation). The sequences of primers were as follows: TGF- β ₁ forward, 5'-GGCAGTGGTTGAGCCGTGGA-3' and reverse, 5'-TGTTGGACAGCTGCTCCACT-3' (590 bp); β -actin forward, 5'-TCGGACGATATGGAGAAGAT-3' and reverse, 5'-ATTGCCGATAGTGATGACGT-3' (240 bp). The PCR cycling conditions were as follows: Initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, and a final elongation step at 72°C for 10 min. The PCR was conducted using an LC480 PCR machine (Roche, Basel, Switzerland). The mRNA expression levels of the target genes were estimated by the optical density values of the bands in the agarose gels.

Western blot analysis. Following harvesting, cells were lysed in cell lysis buffer included in the N-Extract kit (Sigma-Aldrich) and the protein content was determined using a Bradford Protein Assay (Bio-Rad detergent-compatible protein assay; Bio-Rad

Laboratories). Subsequently, equal amounts of protein (50 μ g) were separated by 12% SDS-PAGE and electrotransferred onto a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). Following blocking with 5% non-fat milk in phosphate-buffered saline with 0.05% Tween 20 for 30 min at room temperature, the membrane was incubated with rabbit anti-rat TGF- β ₁ monoclonal antibody (cat. no. 3709; Cell Signaling Technology, Inc.; 1:1,000 dilution) or rabbit anti-rat Akt monoclonal antibody (cat. no. 4685; Cell Signaling Technology, Inc.; 1:2,000 dilution), followed by incubation with peroxidase-conjugated AffiniPure goat anti-rabbit IgG (1:10,000 dilution; cat. no. 111035003; Jackson ImmunoResearch Inc., West Grove, PA, USA) secondary antibody at room temperature for 1 h. Blots were then visualized using an enhanced chemiluminescence detection system (Active Motif, Carlsbad, CA, USA). Quantification of protein levels was performed by determining the relative optical density of the protein bands was using an image analysis system (Image J, version 1.48; National Institutes of Health, Bethesda, MD, USA).

Electrophoretic mobility shift assay (EMSA). Following the indicated treatments, NRK52E cells were assayed for NF- κ B activation using EMSA. Nuclear extracts were hybridized with [³²P]-labeled oligonucleotides containing the sequence GTT GAGGGGACTTTCCAGGC from the NF- κ B promoter or a mutated NF- κ B sequence TCAACTCCCCTGAAAGGG TCCG in binding buffer (Rockland Immunochemicals, Inc., Limerick, PA, USA). These were radiolabeled with [³²P] adenosine triphosphate by T4 polynucleotide kinase for 10 min at 37°C. All reactions were performed in a total volume of 20 μ l containing the binding buffer [10 mM Tris/HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 4% (v/v) glycerol, 5 mM dithiothreitol and 100 μ g/ml bovine serum albumin (Sigma-Aldrich)]. Each sample contained 2 μ l [³²P]-labeled oligonucleotide and 3 μ g poly(dI-dC). After incubation for 15 min at room temperature, samples were electrophoresed on a 5% polyacrylamide gel/0.25X Tris borate EDTA (pH 8.0) (EMD Millipore). For competition experiments, unlabeled oligonucleotides were incubated with extracts for five minutes prior to the addition of radiolabeled probe. After electrophoresis, gels were dried and the protein bands were visualized using Immobilon Western HRP substrate (EMD Millipore) and autoradiographed by exposure to medical X-ray film. The obtained bands were quantified using the Luminescent Image Analyzer-LAS 4000 and Image Gauge software, version 3.1 (Fujifilm Corporation, Tokyo, Japan).

Statistical analysis. Statistical analysis was performed using SPSS, version 17.0 (SPSS, Inc., Chicago, IL, USA). Values are expressed as the mean \pm standard deviation. Significance between groups was determined using one-way analysis of variance. The Q-test was used to analyze differences in the mean values between groups. P<0.05 was considered to indicate a statistically significant difference between values. Each experiment was repeated three times.

Results

Albumin stimulates the expression of TGF- β ₁ mRNA and protein in rat kidney epithelial cells. Analysis of TGF- β ₁

showed that NRK52E cells which were not stimulated with albumin expressed TGF- β_1 mRNA and protein at relatively low levels (Fig. 1). However, TGF- β_1 expression was significantly enhanced by albumin (5-30 mg/ml) in a dose-dependent manner ($P < 0.05$).

Albumin activates NF- κ B protein in rat kidney epithelial cells. Activation of NF- κ B to bind to the promoter region of TGF- β_1 was assessed using an EMSA. As shown in Fig. 2, NRK-52E cells which were not stimulated with albumin exhibited relatively low DNA-binding activity of NF- κ B protein, which was enhanced by albumin (5-30 mg/ml) in a dose-dependent manner with increases of up to five-fold ($P < 0.05$).

MPA reduces albumin-induced Akt phosphorylation. Activation of Akt in NRK52E cells was assessed by western blot analysis using antibodies with specificity for Akt or activated p-Akt only. As shown in Fig. 3A, albumin (5-30 mg/ml) significantly activated Akt in a dose-dependent manner following 12 h of incubation. When cells were starved and treated with albumin (30 mg/ml) for 12 h in the presence of 10 μ mol/l MPA, the phosphorylation of Akt was significantly inhibited ($P < 0.05$) (Fig. 3B), while no marked effects on the levels of total Akt protein were observed.

MPA and Akt inhibitor Ly294002 reduce albumin-induced TGF- β_1 expression. To test the involvement of Akt in albumin-induced TGF- β_1 expression in NRK52E cells, the Akt inhibitor Ly294002 was used. Cells were starved and treated with albumin (30 mg/ml) for 12 h in the presence of 10 μ mol/l MPA or 10 μ mol/l Ly294002. The results showed that Ly294002 as well as MPA significantly inhibited the expression of TGF- β_1 mRNA and protein (Fig. 4A and B). As Akt inhibitor Ly294002 was able to inhibit albumin-induced expression of TGF- β_1 , it was indicated that the Akt pathway is involved in albumin-induced expression of TGF- β_1 . It is further hypothesized that MPA may also exert its effects on albumin-induced expression of TGF- β_1 via the Akt pathway, which requires to be verified in future studies.

Akt inhibitor Ly294002 and MPA inhibit albumin-induced NF- κ B activation. NRK52E cells were starved and treated with albumin (30 mg/ml) for 12 h in the presence of 10 μ mol/l MPA or Ly294002. The stimulatory effects of albumin on the DNA-binding activity of NF- κ B were significantly blocked by Ly294002 ($P < 0.05$). MPA also significantly inhibited albumin-induced NF- κ B activation ($P < 0.05$) (Fig. 5). These results further confirmed that albumin exerts its effects on NF- κ B activity via the Akt pathway; whether MPA inhibits the activation of NF- κ B by albumin via the Akt pathway requires to be confirmed in future studies.

Discussion

Re-absorption of albumin following proteinuria is closely correlated with the imbalance of proliferation/apoptosis and phenotypic differentiation of renal tubular epithelial cells, as well as the infiltration of inflammatory cells. Injured renal tubular epithelial cells cultured *in vitro* have a high rate of proliferation and secrete large amounts of extracellular

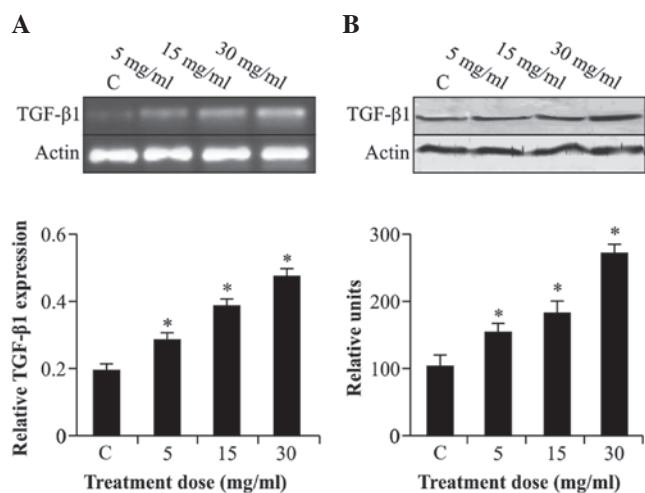


Figure 1. Effects of albumin on the expression of TGF- β_1 at the (A) mRNA and (B) protein level. Expression was significantly enhanced by albumin (5-30 mg/ml) in a dose-dependent manner. Expression levels were normalized to β -actin and values are expressed as the mean \pm standard deviation ($n=3$). * $P < 0.05$ vs. C. C, control; TGF, transforming growth factor.

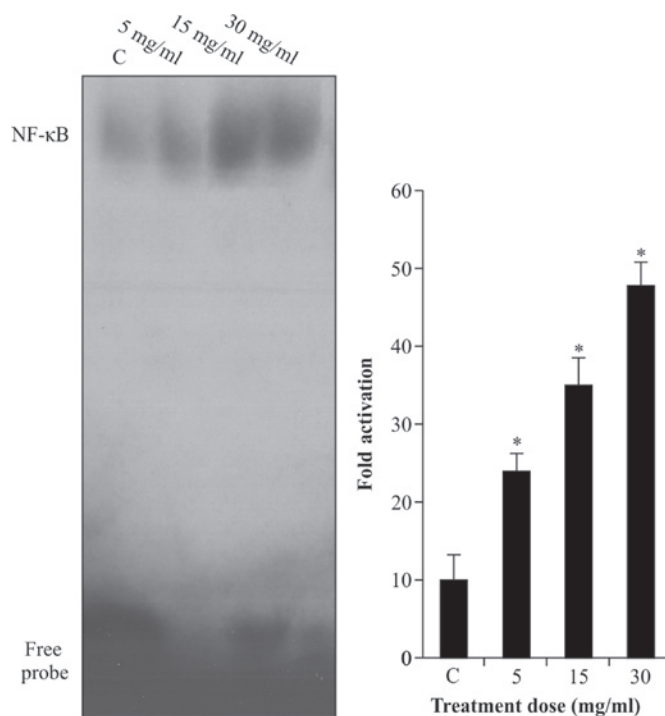


Figure 2. Effects of albumin on the binding of NF- κ B protein to the promoter region of the transforming growth factor beta 1 gene. An electrophoretic mobility shift assay demonstrated that albumin stimulated the DNA-binding activity of NF- κ B in cultured NRK52E cells in a dose-dependent manner (5-30 mg/ml). Values are expressed as the mean \pm standard deviation ($n=3$). * $P < 0.05$ vs. C. C, control; NF, nuclear factor.

matrix, which results in fibrosis (1). Simultaneously, the injured renal cells transdifferentiate into muscular fibroblasts which secrete large amounts of growth factors, thereby amplifying the local immunoinflammatory reaction and accelerating the process of fibrosis (11,12). Proteinuria is a well-known exacerbating factor in renal tubular interstitial disease (13). Renal interstitial fibrosis is a common pathological process in progressive renal diseases, which leads to

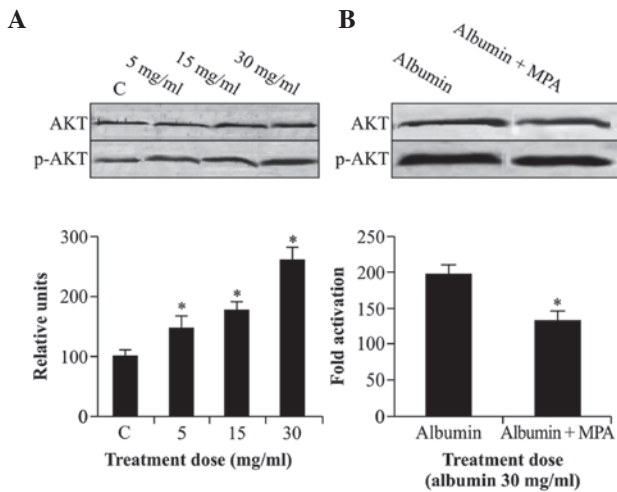


Figure 3. Effects of MPA on Akt phosphorylation. (A) Activation of Akt was assessed by western blot analysis using p-Akt-specific antibodies. * $P < 0.05$ vs. C. (B) The phosphorylation of Akt was inhibited by 10 μ M MPA (* $P < 0.05$ vs. albumin only), while total Akt was not affected. Values are expressed as the mean \pm standard deviation ($n = 3$). C, control; MPA, mycophenolic acid; p-Akt, phosphorylated Akt.

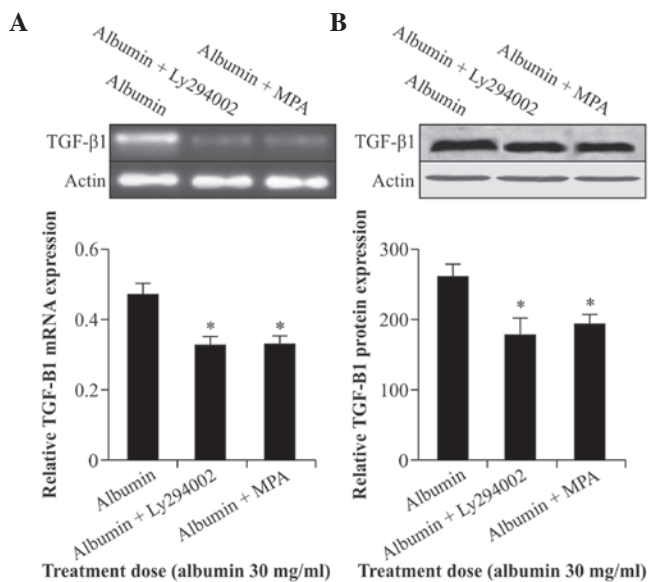


Figure 4. Effects of Ly294002 and MPA on albumin-induced TGF- β ₁ expression. Ly294002 and MPA significantly inhibited the (A) mRNA and (B) protein expression of TGF- β ₁. β -actin was used as an internal standard. Values are expressed as the mean \pm standard deviation ($n = 3$). * $P < 0.05$ vs. albumin. TGF, transforming growth factor; MPA, mycophenolic acid.

functional deterioration of renal cells and eventual loss of renal function (14-17).

MPA, the active metabolite of MMF, is a potent, non-competitive and reversible inhibitor of inosine-5'-monophosphate dehydrogenase, the rate-limiting enzyme for *de novo* purine synthesis. MPA has an effect on cell growth and chemokine release of tubular epithelial cells, and these effects are dependent on the inhibition of cellular guanosine production. However, limited data are available on the effects of MPA on renal tubular epithelial cells (4,18).

TGF- β ₁ is a fibrogenic and inflammatory cytokine with a central role in the pathogenesis of renal fibrosis (19). It has

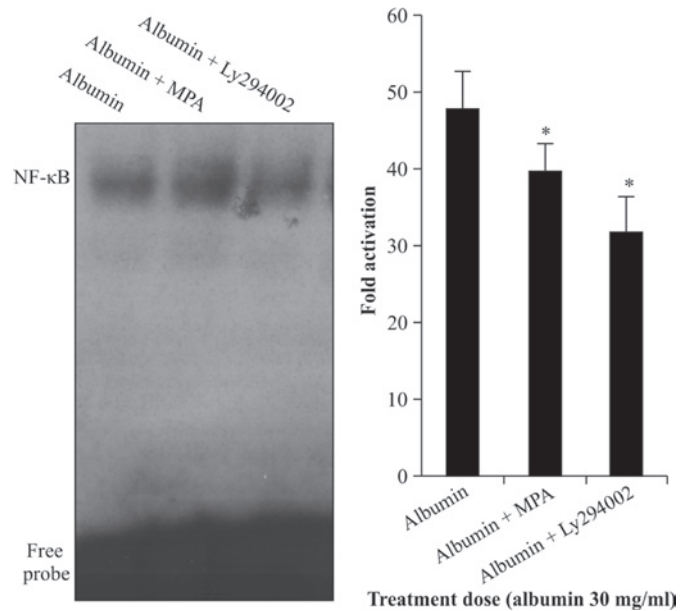


Figure 5. Effects of Ly294002 and MPA on albumin-induced binding of NF- κ B to the promoter region of transforming growth factor beta 1. An electrophoretic mobility shift assay showed that the stimulatory effect of albumin on NF- κ B was blocked by Ly294002. MPA also significantly inhibited albumin-induced NF- κ B activation. Values are expressed as the mean \pm standard deviation ($n = 3$). * $P < 0.05$ vs. albumin only. NF, nuclear factor; MPA, mycophenolic acid.

been demonstrated that treatment with TGF- β ₁ antibody restrained the function of TGF- β ₁ to reduce the degree of tubular fibrosis (20). A further study showed that intraperitoneal injection of TGF- β induced renal fibrosis in mice (21). In the present study, albumin was shown to stimulate the expression of TGF- β ₁ in tubular epithelial cells in a dose-dependent manner, indicating that albumin triggers mechanisms in renal tubular epithelial cells leading to fibrotic injury. This mechanism may be the underlying reason for proteinuria causing renal interstitial fibrosis. Furthermore, the present study demonstrated that MPA inhibited TGF- β ₁ expression, thereby potentially preventing fibrotic injury.

The promoter region of the TGF- β ₁ gene contains NF- κ B-binding sites. NF- κ B is a transcription factor which consists of a p50 and a p65 sub-unit. NF- κ B regulates the production of pro-inflammatory mediators in cellular inflammation. Activation of NF- κ B drives renal inflammation and fibrosis (22). In the resting stage, NF- κ B exists in its inactive form in the cytoplasm; however, it becomes activated when cells are stimulated (23). The present study revealed that albumin overload can stimulate the binding activity of NF- κ B to the promoter region of TGF- β ₁. Furthermore, MPA was able to inhibit albumin-induced activation of NF- κ B protein.

Akt is a serine/threonine protein kinase which is mainly responsible for the initiation of biological signal transmission by PI3K. Akt is in the central axis of the Akt/PI3K pathway, with its functions including cell cycle regulation, induction of apoptosis, and the participation in numerous important physiological and pathological process, including angiogenesis, telomerase activity and malignant characteristics of cells (24). Akt also regulates cell activation and proliferation through NF- κ B (25). It has been reported that Akt increases the

phosphorylation of inhibitor of NF- κ B (I κ B) and reduces I κ B protein synthesis to activate the NF- κ B. Abnormal activation of Akt has an important role in processes leading to renal fibrosis (26). The present study suggested that albumin significantly enhanced the level of Akt activation in NRK52E cells following 12 h in a dose-dependent manner. This result suggested that Akt phosphorylation has an important role in processes of renal tubular epithelial cell injury. Treatment with Ly294002, a specific inhibitor of Akt, inhibited the expression of TGF- β_1 and activation of binding of NF- κ B to the promoter region of TGF- β_1 . This result indicated that albumin induced the synthesis of TGF- β_1 and the activation of NF- κ B in tubular epithelial cells partly through the phosphorylation of Akt.

The present study showed that albumin significantly increased TGF- β_1 expression and NF- κ B activation. Furthermore, MPA and Ly294002 inhibited TGF- β_1 expression and NF- κ B activation. In addition albumin significantly enhanced Akt activation, which was inhibited by MPA. The observation that the Akt inhibitor effectively inhibited albumin-induced TGF- β_1 expression and NF- κ B activation leads to the hypothesis that MPA exerts its anti-fibrotic effects, at least partially, by inhibiting Akt activation; however, this remains to be experimentally verified in future studies.

In conclusion, the present study demonstrated that MPA inhibited albumin-induced TGF- β_1 expression and the binding of NF- κ B to the promoter region of TGF- β_1 , possibly through inhibiting Akt activation.

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