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Original Article

The protective effects of ischemic postconditioning on renal tubular epithelial Epithelial-mesenchymal transition

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Abstract: Renal tubular Epithelial-mesenchymal transition (EMT) plays an important role in the process of renal fibrosis. The aim of this study is to observe the effects of ischemia post-conditioning on renal tubular Epithelial-mesenchymal transition in vitro. A valid in vitro ischemia post-conditioning model was established by using NRK-52E cell line. The ischemic postconditioning (IPO) model was established by placing the cells in an ischemic condition for 3 hours and then followed by exposing the cells to three cycles of a reperfusion state for 10 minutes and an ischemic state for another 10 minutes. Flow cytometry and Hoechst were used to gain access to the apoptosis that took place. The protein expression levels of α-SMA, TGF-β, CTGF and Ecadherin were detected by western blot. The simulating I/R injury results in severe injury in NRK-52E cells as evidenced by increased apoptotic index, which may be significantly attenuated by IPO treatment applied before the abrupt reperfusion ($P<0.05$ vs I/R group). Meanwhile, I/R increase the expression levels of α-SMA, TGF-β, CTGF, and decrease the expression levels of E-cadherin. However, IPO inhibts the expression changes of these proteins. The results offer evidence that I/R injury causes cell apoptosis, and IPO can effective to attenuate renal cell apoptosis. Meanwhile, IPO could inhibit the occurrence of EMT potentially mediated by decreasing the expression of α-SMA, TGF-β, CTGF and protetecting the expression of E-cadherin.

Keywords: Epithelial-mesenchymal-transition (EMT), fibrosis, ischemic postconditioning, ischemic reperfusion

Introduction

Renal ischemia/reperfusion (I/R), which is an important cause of renal dysfunction. Moreover, severe renal I/R not only cause renal failure, but also leads to chronic tubulointerstitial fibrosis, which is a necessary part of the various causes of kidney disease progressed to end stage renal disease. During the process of renal fibrosis, the renal tubular Epithelial-mesenchymal transition (EMT) plays a major role.

The process of EMT in embryonic development is for primitive epithilial cells to form the mesoderm and primitive neuroepithelial cells to form neural crest cells. The conversion of epithelial cells to mesenchymal cells is fundamental for embryonic development and profound phenotypic changes may occur including the loss of cell-cell adhesion, the loss of cell polarity, and the acquisition of migratory and invasive properties [1, 2]. In the field of cancer cell biology, EMT is an important molecular mechanism of tumor invasion and metastasis [3]. However, it is worth noting that the cancer cell of EMT does not lead to the formation of metastases of fibroblasts and only epithelial cells acquire mesenchymal characteristics, which had significant difference from the ordinary myofibroblasts. Over the course of the past decade, emerging evidence suggests that EMT is a source of myofibroblast recruitment in the fibrosis process [4].

This model is often referred to as the type 2 EMT, considered that under the condition of chronic injury, the special shape of mature epithelial cells disappear, through the tubular basement membrane into the interstitium, and eventually “differentiation” as the myofibroblasts phenotype, with the capacity of synthesis and deposition of extracellular matrix. There

are a series of studies to support this hypothesis: Firstly, under the culture conditions of Transforming Growth Factor-beta 1 (TGF-β1) in vitro, the original tubular epithelial cells can experience phenotypic transformation. The characteristics of this transition include the loss of epithelial features such as E-cadherin, zonula occludens-1 (ZO-1), cytokeratin, and activated the markers of interstitial or myofibroblast cells including the expression of α-smooth muscle actin (α-SMA), vimentin, fibroblast-specific protein-1 (FSP-1) and interstitial matrix components [5]. Secondly, Double-labeling immunohistochemistry co-localization of epithelial and mesenchymal cell markers to detect kidney damage showed simultaneous expression of two intermediate cell markers, this indicating the presence of EMT. Finally, the most important and a landmark study published in 2002, which proposed using the Cre/Lox technology and the analysis of cell lineage to trace the renal tubular epithelial fate in the process of renal fibrosis, for the first time in vivo, proved the existence of EMT in renal tubular epithelium [6]. However, our primary study showed that IPO may inhibit the EMT of renal tubular epithelial which induced by ischemic renal fibrosis [7]. In the study α-SMA and TGF-β1 expression were inhibited by IPO in vivo. Therefore, the present study utilized an in vitro model we preestablished to evaluate the effects of ischemic postconditioning (IPO) on the renal tubular epithelial-mesenchymal transition, to verify whether the IPO in vitro can inhibit EMT in the renal tubule.

Materials and methods

Cell culture

The renal tubular epithelial cell line, NRK-52E cells, was purchased from Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The cells were cultured on culture dishes and gassed with 95% air/5% CO_2 and maintained at pH 7.4 and 37°C. The medium was changed once every two days, and the cells were used for experiments at day 10 after seeding. All experiments were performed with cells that were cultured in serum-free medium for 24 hours prior to the experiments. Cells were seeded on 6 well plates or culture dishes as appropriate.

In vitroischemic postconditioning model

The complete medium is replaced with serum free DMEM one day before the experiment to synchronize the cells. The preparation of control buffer and ischemic buffer refference method for the preparation of C Sauvant [8]. Then, the cells are randomly divided into three treatment groups as follows: 1) Control group: NRK-52E cells are first incubated in 1 mL control buffer ($NaHCO_3$ 24.0 mM, Na_2HPO_4 0.8 mM, NaH_2PO_4 0.2 mM, NaCl 86.5 mM, KCl 5.4 mM, $CaCl_2$ 1.2 mM, $MgCl_2$ 0.8 mM, HEPES 20 mM; pH adjustment to 7.4 with 1N NaOH) under normoxic conditions (95% air-5% CO_2) for 3 hours (h), and then are incubated in 2 mL fresh complete DMEM medium (DMEM-10% FBS-streptomycin 100 g/ml and penicillin 100 U/ml) under normal conditions (95% air-5% CO_2) for 24 h; 2) Ischemic and reperfusion injury (I/R) group: NRK-52E cells are washed with 1 mL serum free DMEM medium pH 7.4, following with 1 mL sugar free DMEM medium pH 7.4, and then are incubated in 1 mL ischemia buffer ($NaHCO_3$ 4.5 mM, Na_2HPO_4 0.8 mM, NaH_2PO_4 0.2 mM, NaCl 106.0 mM, KCl 5.4 mM, $CaCl_2$ 1.2 mM, $MgCl_2$ 0.8 mM, MES 20 mM; pH adjustment to 6.6 with 1N NaOH) under hypoxic conditions (0.5% O_2-5% CO_2-94.5% N_2) for 3 h. After this phase, the serum free and sugar free DMEM medium is replaced with complete DMEM medium to simulate reperfusion, followed by 3 h, 6 h, 12 h and 24 h in normal conditions. 3) Ischemic postconditioning by "adding" medium group (IPO): NRK-52E cells are first incubated in 1 mL ischemia buffe under hypoxia conditions for 3 h. After this phase, the culture dishes are transferred to a normoxic conditions incubator in a humidified atmosphere with adding another 0.5 mL fresh complete DMEM medium for 10 min. Subsequently, the cells are returned to hypoxia conditions for 10 min. The postconditioning cycle is repeated three times and is described as IPO. Finally, the cells are incubated in 2 mL fresh complete DMEM medium under normoxic conditions for 3 h, 6 h, 12 h and 24 h.

Fluorescein isothiocyanate (FITC)-conjugated annexin-V-propidium iodide (PI) dual staining

The 5×10^5 NRK-52E cells were cultured in a 6-well plate with 2 mL serum-free medium. 24

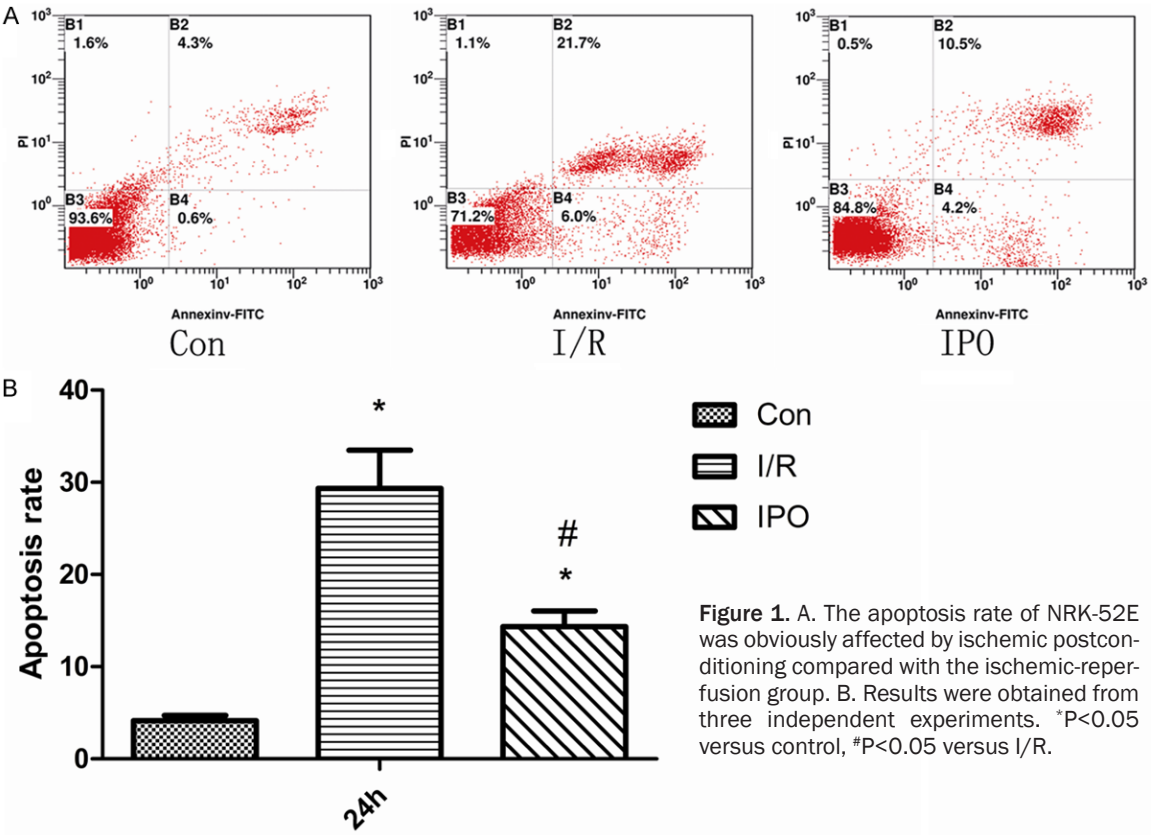

Figure 1. A. The apoptosis rate of NRK-52E was obviously affected by ischemic postconditioning compared with the ischemic-reperfusion group. B. Results were obtained from three independent experiments. *P<0.05 versus control, #P<0.05 versus I/R.

h later, all cells were processed in according with the above grouping. After an additional 24 h culture, NRK-52E cells were collected and stained with FITC-conjugated annexin V and propidium iodide according to the steps given in the apoptosis detection kit, and testing completed within an hour after dyeing. Then we performed flow cytometry for apoptosis analysis (Apoptosis Kit, BD Pharmingen, Germany).

Hoechst 33342 assay

Exponentially growing cells are plated in 6-well plates at a density of 2×10^5 cells/well and cultured for 72 h. Following simulated I/R and IPO procedures, cells are fixed for 8 min with the precooled (-20°C) formaldehyde and acetone solution (1:1, v/v) and washed with PBS 3×3 min, followed by staining with Hoechst33342 solution (50 mmol/L) at 37°C in darkness for 5 min. Apoptotic cells are observed and images are taken using fluorescence microscope (OlympusBX51, Tokyo, Japan), with excitation wavelength of 350 nm and emission wavelength of 460 nm.

Protein analysis by western blot

The protein expression levels of α-SMA, TGF-β, CTGF and E-cadherin were examined by western blotting. Briefly, proteins were extracted from NRK-52E, subjected to SDS-PAGE on 10% polyacrylamide gels (20 μg/lane), and then electrotransferred to nitrocellulose membrane. The membranes were blocked with 5% nonfat milk in TBST buffer (10 mmol/L Tris-HCl, 0.15 mol/L NaCl, and 0.05% Tween 20, pH 7.2) and incubated with the following rabbit polyclonal primary antibodies. Subsequently, the membranes were incubated with secondary horseradish peroxidase-conjugated anti-rabbit IgG antibody. The membrane was washed extensively with TBST, and the immunoreactive bands were detected with ECL-detecting reagents.

Statistical analysis

Assays were performed in triplicate. Quantitative data are presented as the mean ± SEM. Statistical analyses were performed with the GraphPad Prism software 5.0 (GraphPad Software, Inc., SanDiego, CA). For determing the

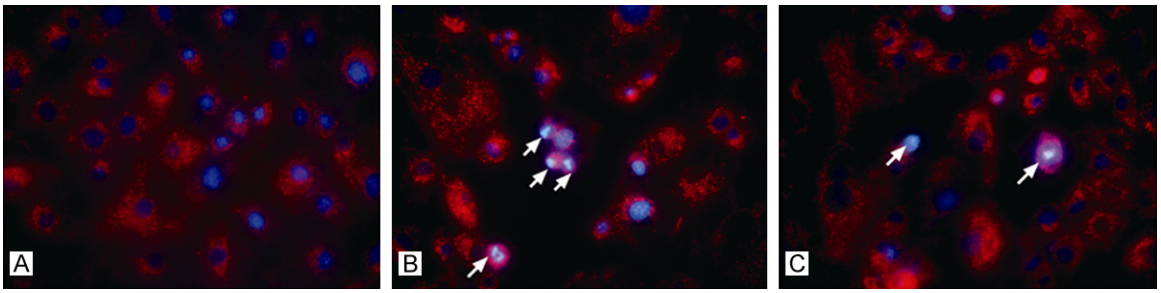

Figure 2. Effect of postconditioning on ischemia/reperfusion induced apoptosis of NRK-52E cells with Hoechst 33342 staining. A. Control, NRK-52E cells culture in control medium under normal condition. B. I/R, cells cultured in ischemic condition and then reperfusion for 24 h. C. IPO, cells cultured in ischemic condition, immediately for three cycle postconditioning and then reperfusion up to 24 h. ("→" Indicates the apoptotic cells. All fluorescence photomicrographs original magnification ×200).

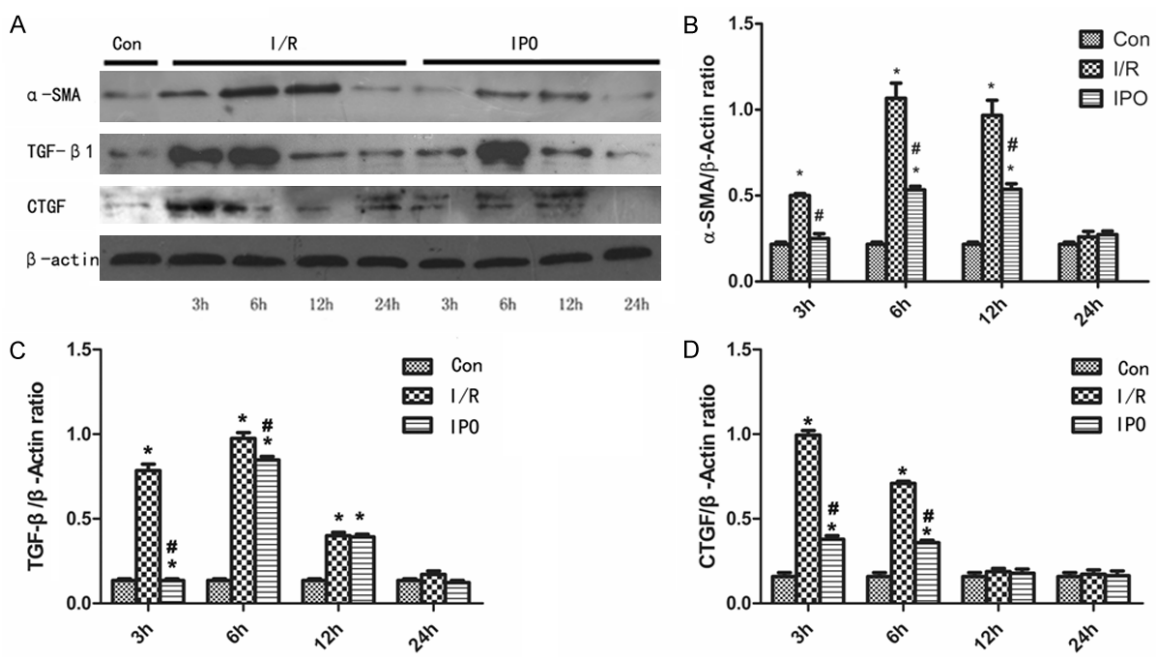

Figure 3. The IPO promotes protective effects on NRK-52E cells and inhibits the expression levels of α-SMA, TGF-β1 and CTGF in the three groups of NRK-52E cells line (*P<0.05 versus control, #P<0.05 versus I/R. n=4).

numbers of apoptotic cells, comparisons among groups were compared using one-way ANOVA3 Student-Newman-Keuls test. P<0.05 was used to determine statistical significance.

Results

Evaluation of apoptosis rates by fluorescein isothiocyanate (FITC)-conjugated annexin-V-propidium iodide (PI) dual staining

NRK-52E subjected to synchronization was treated in accordance with the above grouping. After 24 h of culture, NRK-52E were collected and stained with FITC-conjugated annexin V and propidium iodide for detecting apoptosis. As shown in **Figure 1**, the rate of apoptosis of the control group was significantly lower than the I/R and IPO groups (P<0.05). Compared with both I/R and IPO groups, the rate of apoptosis of I/R group is significantly higher than IPO (P<0.05).

Hoechst 33342 staining

Effects of postconditioning on the I/R induced cell apoptosis are assayed by Hoechst 33342 staining. As shown in **Figure 2**, few apoptotic

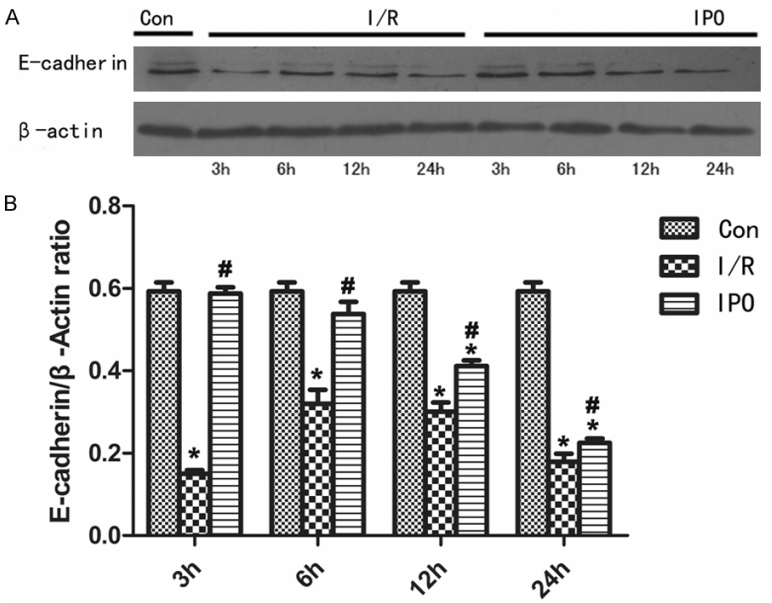

Figure 4. The IPO promotes protective effects on NRK-52E cells and the expression levels of E-cadherin in the three groups (*P<0.05 versus control, #P<0.05 versus I/R. n=4).

cells are present in the normal group. As expected, there are many apoptotic (Hoechst 33342-positive) cells in the I/R group and in the IPO group. However, compared with the I/R group, fewer apoptotic cells are observed in the IPO group.

Protein expression

The results of protein extracts of α-SMA, TGF-β, and CTGF in various cell groups of NRK-52E were subjected to Western Blot Analysis are shown in **Figure 3**. Same amount of the extracted protein (20 μg/lane) was loaded on the same gel. β-actin was used as positive control. After treatment of 3 h, 6 h, 12 h and 24 h at different time points, the expression of α-SMA in the IRI group increased at 3 h, 6 h, 12 h, and then decreased. However, in contrast to the IRI group, the expression levels at 3 h, 6 h, 12 h were significantly suppressed in IPO group. The expression of TGF-β in the IRI group increased at 3 h, 6 h, through the reperfusion stage, and then decreased. Meanwhile, the IPO group expressions similar to the IRI group, although expression levels at 3 h, 6 h were significantly suppressed. Interestingly, the expression mode of CTGF in IRI group is similar to TGF-β, the expression levels at 3 h, 6 h were significantly increased, then decreasing. Compared with the control group, the IPO group expression did not change significantly at each time point. In this study, we also evaluated the expression of E-cadherin in NRK-52E cells, as shown in **Figure 4**. The expression levels in the IRI group was decreased at 3 h, while IPO inhibit this change, and the expression levels of IPO had no significant differences compared with the normal control group.

Discussion

Renal interstitial fibrosis, characteristics including the heavily extracellular matrix aggregation in renal interstitial and tubular, is a key process of kidney disease. Meanwhile, renal interstitial fibrosis is the result of combined action of a variety of reasons, and is the main pathological basis of end-stage renal disease. When slight renal ischemia-reperfusion occurs in clinic, the renal function and pathological changes will gradually return to normal with the elapse of time. However, when severe renal IRI occurs, the renal tubular changes are irreversible and subsequently manifested as tubular fibrosis, thus resulting in a decline in renal tubular concentration function and renal dysfunction [9-11]. The Previous view was that, during the period of reperfusion, the renal tubular had powerful proliferation ability to repair injury and IRI can be completely reversed [12]. However, recently researchers have found that the capacity of proliferation and repair of renal tubular epithelial cells is limited, which depends on the survival number of tubular cells and wether the basment membrane is intact. Renal tubular injury is irreversible when the extent of damage exceeds a critical value, and the structure and function of renal tubular injury cannot be fully recovered [13]. Some researchers have found that the moderate fibrosis lesions of renal tubular interstitial disease does not cause an abnormal rise in blood urea nitrogen and serum creatinine, but may destroy the reabsorption function of urine thus causing increased urination at night and proteinuria [13].

The mark of myofibroblasts is α-SMA, and it is the activation forms of fibroblasts, which is also a major source of extracellular matrix collagen ingredients [14, 15]. The α-SMA only expressed in arterial smooth muscle cells in normal renal tissues. However, in the present study, we confirmed the expression of α-SMA in the IRI group increased at 3 h, 6 h and 12 h. However, in contrast to the IRI group, the expression levels at 3 h, 6 h and 12 h were significantly suppressed in IPO group. Thus, the present study demonstrated that ischemia-reperfusion injury in vitro can also induce the expression of α-SMA and induce renal tubular epithelial cells to acquire mesenchymal polarity as well as the occurrence of EMT.

During the process of renal interstitial fibrosis, the TGF-β1 was considered to be an important one in many fibrogenic factors. The main function of TGF-β1 was to increase the synthesis of extracellular matrix (ECM), inhibit its degradation and up-regulation of integrin matrix adhesion molecules [16, 17]. In multiple downstream signaling pathways of TGF-β, the Smad signal pathway plays an important role in the process of multiple organ fibrosis, Smad 2 was an pivotal molecule to initialize the signal transduction pathway [18]. A large number of investigate of TGF-β1/Smad pathway was found that, by antagonism the activation of TGF-β1 can alleviate renal interstitial fibrosis [17]. Yasuda K et al found that through upregulate the expression of TGF-β1 can increase renal tubular extracellular matrix, indicating the increased continuously of TGF-β1 has the potential to promote fibrosis [19]. In this experiment, we confirmed that the expression levels of TGF-β1 in the IRI group were significantly increased at 3 h, 6 h reperfusion, while its expression in the IPO group was significantly suppressed, shows that IPO can inhibit the expression of TGF-β1 in renal tubular epithelial cells of an ischemia-reperfusion model.

Connective tissue growth factor (CTGF) is matrix protein, plays a crucial role in the process of pathological fibrosis. In vivo and in vitro experiments have proved that, CTGF expressed in renal tubular epithelial cells, suggesting its involvement in the procedure of renal fibrosis and the course of EMT. The CTGF is a potential biomarker of EMT in chronic allograft nephropathy [20, 21]. In the present study, we confirmed that the expression levels of CTGF in IRI group were obviously enhanced at point of 3 h, 6 h reperfusion, while its expression in IPO group were evidently attenuated, indicating that IPO can restrain the expression of CTGF in renal tubular epithelial cells of an ischemia-reperfusion model.

E-cadherin, which expressed in well-differentiated and polarized epithelial cells, such as the renal tubular epithelium, is a kind of adhesion protein [22]. Under pathological conditions, when renal tubular epithelial cells lose their E-cadherin, is when epithelial cells are undergoing the process of EMT [23]. In this experiments, the expression of E-cadherin in IRI group were decreased at 3 h, while the expression of E-cadherin in IPO groups had no significant changes compared with the normal control group.

In summary, based on the above study, we demonstrated that IPO can relieve renal injury caused by ischemia-reperfusion, and protect the pression of E-cadherin, effectively inhibiting the expression of α-SMA, CTGF and TGF-β1 in renal tubular epithelial cell line NRK-52E cells, indicating the possibility to inhibit the occurrence of EMT, its protective mechanism may be involvement of the TGF-β1/Smad signal pathway, thus decreasing the tubulointerstitial fibrosis. However, these findings remain to be confirmed by future studies. The current study shows that IPO improved the ability of renal tubular epithelial cells to tolerate ischemic-reperfusion injury, particularly to decrease α-SMA, TGF-β and CTGF expression, and protecting the expression of E-cadherin, thus experimental studies in vitro model revealed that IPO may effectively inhibit the occurrence of EMT.

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Disclosure of conflict of interest

None.

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