Full Length Research Paper

Endothelial protein C receptor in renal tubular epithelial cells and influencing factors

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The endothelial protein C receptor (EPCR) plays an important role within the protein C pathway in regulating coagulation and inflammation. It was reported that EPCR was expressed in large vessels, placenta, heart, liver and lung endothelial cell. However, there are a few studies concerned about renal epithelial cells. This study aims to investigate EPCR expression in renal tubular epithelial cells and related influencing factors. EPCR expression was assessed by flow cytometry in renal tubular epithelial cells. The effects of some reagents (high glucose, tumor necrosis factor–α and interleukin-1β) were measured by RT-PCR. The results showed that renal tubular epithelial cells had the high expression of EPCR level. High glucose, tumor necrosis factor–α and interleukin-1β might reduce EPCR expression. And troglitazone could significantly improve the inhibition. In conclusion, we found EPCR expression in renal tubular epithelial cells *in vitro*. Some factors such as high glucose, tumor necrosis factor–α and interleukin-1β can impact on EPCR. However, troglitazone had protective effects of EPCR on injured cells.

Key words: Endothelial protein C receptor, renal tubular epithelial cell, troglitazone, tumor necrosis factor-α, interleukin-1β; high glucose.

INTRODUCTION

A large number of basic and clinical studies have confirmed the close correlation that exists between the protein C system and anti-inflammatory effects (Joyce et al., 2001; Van de Water et al., 2001; Tsuneyoshi et al., 2001; Esmon et al., 2003). Activated protein C (APC) has protective effects on inflamed endothelial cells and shows a direct link to the reduction of mRNA levels in endothelial nuclear factor-kappa B (NF-KB). This will result in the inhibition of the expression of cell surface adhesion molecules and the reduction in the synthesis and release of inflammatory cytokine, inhibiting neutrophil activation and extravasation in damaged tissue parts. Endothelial protein C receptor (EPCR) was first reported by Fukudome in 1994, expressed in large vessels, placenta,

plasma in soluble form (Fukudome et al., 1994). Generally, APC activity can be enhanced when combined with EPCR, which participates in APC-mediated anti-inflammation and anti-apoptosis (Laszik et al., 1997; Gu et al., 2000; Sturn et al., 2003; Nan et al., 2005; Liaw et al., 2000). In this study, we observed EPCR expression in renal tubular epithelial cells (RTECs) *in vitro*. Meanwhile, we also detected the effects of various factors such as high glucose, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) on EPCR and also, whether troglitazone had a certain antagonistic effect on the stated influencing factors. These tests were performed to further clarify the significance and the protective effects of EPCR on injured RTECs.

MATERIALS AND METHODS

Cell culture

RTECs were frozen cell lines donated by the Kidney Institute of

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PLA General Hospital of Nanjing Military Region. To recover cells,they were cultured in RPMI-1640 medium containing 15% calf serum (Gibco Inc, USA) at 37℃, 5% CO_2. The medium was changed once every 2 to 3 days; passages were conducted based on appropriate density. RTECs at a density of $1×10^5$ cells/ml were inoculated in a 6-well plate, cultured in RPMI-1640 medium containing 15% calf serum (Sijiqing Inc, Hangzhou) at 37℃, 5% CO_2 for 24 h. Under high power microscope, anchorage-dependent cell growth was observed, presenting 80 to 90% confluence. It was followed by the replacement of serum-free RPMI-1640 medium with complete medium and was incubated overnight at 37℃, 5% CO_2.

The measurement of EPCR expression by flow cytometry

The cytomics FC 500 flow cytometer (Beckman Coulter) was applied to detect the expression of membrane EPCR. Cells grown to 80% confluence were digested with 0.25% trypsin (DIFCD Inc.), collected to ensure that the cells reached $1×10^6$ cells/ml and washed with 1×PBS three times; 200 µl 1×PBS was added to each tube, resuspended, then evenly divided into two tubes. In one tube, 20 µl of mouse anti-human EPCR first antibodies (1 µg/20 µl) were added. In the second tube, 20 µl 1×PBS was added as a control responded in a 4℃ wet box for 1 h, centrifuged and then the supernatant was discarded and washed with 1×PBS three times. Next, 250 µl 1×PBS was added to each tube to be resuspended; 10 µl FITC-goat anti-mouse secondary antibodies (Immunotech, Inc.) (1 µg/10 µl) were added into each tube, reacted for 30 min in the dark. After being centrifuged, the supernatant was discarded, washed with 1×PBS three times; 250 µl 1×PBS was added to each tube, re-suspended and detected by flow cytometry.

Effects of several reagents

Four reagents were used on RTECs, respectively, to observe the effects of different reagents in different concentrations on EPCR gene expression. The methods are as follows: RTECs were cultured in low-serum (containing 3% calf serum, the following are the same) RPMI-1640 medium containing D-glucose for 24 h, EPCR mRNA expression was observed at different concentrations of D-glucose (5, 10, 30 and 50 mmol/l). The non-reagent treated group, under the same conditions, was used as the control group. Besides glucose, TNF -α and IL-1β (2, 5, 10 and 20 mmol/l, respectively) (Shanghai Jingmei Bio-engineering Co., Ltd.) also acted on the cultured cells according to the mentioned method. And their effects on EPCR gene expression were observed.

In this experiment, it was also observed whether troglitazone (Cayman, Ann Arbor, MI) had a protective effect on the EPCR gene when impacted by the reagents stated earlier. The specific methods are as follows: cells grown to 80% confluence were cultured in media containing 50 mmol/l D-glucose, 20 ng/ml TNF-α, and 20 ng/ml IL-1β, respectively, for 24 h; these were used as the positive control group. In the experimental group, RTEC cell lines were cultured in medium containing troglitazone at concentrations of 0, 5, 10 and 20 µmol/l, respectively, for 12 h. Then, we replaced with the new media containing the various reagents described earlier. Meanwhile, the non-reagent treated group was used as the control. Each experimental group had 5 duplicate wells and each experiment was performed in triplicate.

EPCR expression detected by real-time PCR

Primer sequences are as follows: β-actin primers Forward: 5'CTCGCGCTACTCTCTCTTTC3', Reverse: 5'CAGTCTCGATCCCACTTAA3' EPCR primers Forward: 5'ATTGCTGCCGATACTGCT3' Reverse:

5'AGAGGAAAGGCCAAGGTC3', Reaction system MJ Research Option2TM (MJ Inc.): 5×buffer 5 µl, Mg2 + (250 Mm) 0.25 µl, dNTP (10 Mm) 0.75 µl, Taq enzyme (250 U/50 ul) 0.25 µl, forward primer (10 Mm) 0.5 µl, reverse primer (10 mM) 0.5 µl, Evagreen (10 mM) 0.5 µl, double-distilled water 15.45 µl, cDNA 2 µl, a total of 25 µl system. Reaction conditions: pre-denatured at 95℃ for 5 min, 95℃,15 s → 58℃,25 s → 72℃, 25 s → 78℃, 0.1 s; reading plate; 50 cycles; 60 to 96℃, 0.3 s melting curve; 72℃ for 5 min; 4℃. Formula:

Relative amount = 2 - $\triangle$C (T)
$$(1)$$
$\triangle$C (T) = target gene C (T) - internal reference gene C (T)

Experimental group: relative amount in control group: 2 - $\triangle\triangle$C (T) (2)
$\triangle\triangle$C (T) = experimental group $\triangle$C (T) - control group $\triangle$C (T)

Statistical analysis

Each experimental step was repeated for at least 3 times, each intervention group had five duplicate wells and the results obtained were consistent. Mean ± standard deviation (± SD) of the final results of each experiment was taken as the statistics. ANOVA or t test was used for statistical analysis, $P < 0.05$ was considered statistically significant.

RESULTS

Flow cytometry

The flow cytometry result showed that high expression, 97.9%, of EPCR in the RTECs. It is shown in Figure 1.

RT-PCR

EPCR mRNA levels in cell after 24 h are shown in Figure 2. EPCR mRNA expression in RTECs was significantly decreased by high glucose, especially at the D-glucose concentration of 30 and 50 mmol/l ($P < 0.05$) and showed a significant dose-response relationship.

Changes in EPCR mRNA after 24 h are shown in Figure 3. 2 ng/ml TNF-α could significantly reduce EPCR mRNA expression ($P < 0.05$), EPCR mRNA expression and TNF-α dose presented a dose-effect relationship.

EPCR mRNA expression levels after 24 h are shown in Figure 4. In relation to the increasing IL-1β dose, EPCR mRNA expression was significantly reduced. EPCR mRNA expression began to decrease at 10 ng/ml ($P < 0.05$), but the dose-effect relationship in the range of experimental dose was not satisfactory.

As previously confirmed, EPCR mRNA expression was significantly inhibited in RTECs stimulated with 50 mmol/l D-glucose, 20 ng/ml TNF-α and 20 ng/ml IL-1β for 24 h. The results of the cells stated earlier are protected by troglitazone at concentrations of 0, 5, 10 and 20 µmol/l, respectively. Detected in each group are shown in Figure 5
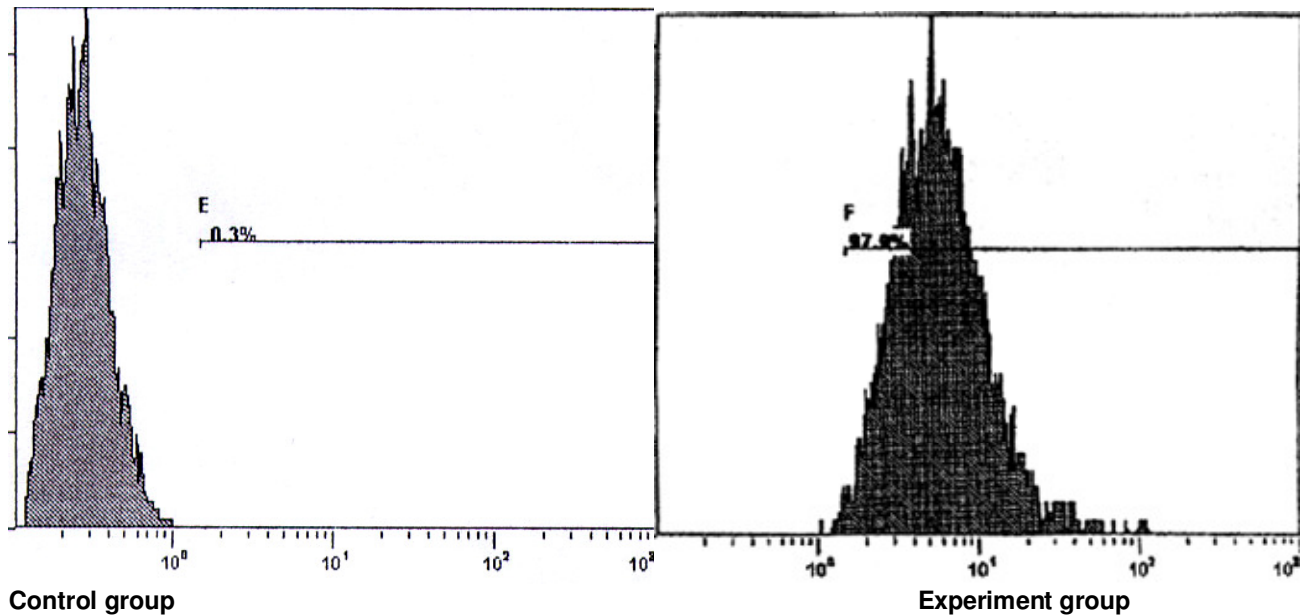
Control group   **Experiment group**

Figure 1. The measurement of EPCR expression by flow cytometry. High expression of EPCR can be discovered in experiment group (RTECs with EPCR antibodies).
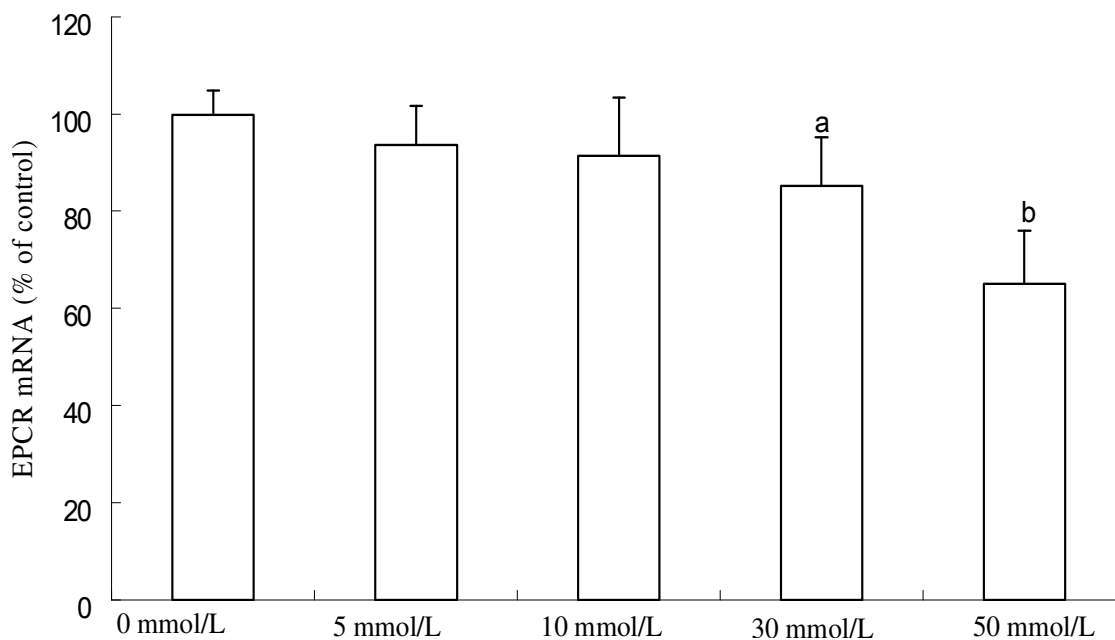

Figure 2. Effects of different concentrations of D-glucose on EPCR expression. The mRNA level of EPCR was significantly decreased. A, $p < 0.05$; b, $p<0.01$, compare with the control (0 mmol/L).

The pretreatment of appropriate concentration of troglitazone on RTECs could reverse-at different degrees-the reduction effect of high glucose, TNF-α and IL-1β on EPCR mRNA expression ($P < 0.05$). 5 μmol/l troglitazone could just improve the inhibition of drugs to EPCR ($P < 0.05$). The protection of troglitazone from inhibition of high glucose and IL-1β to EPCR showed a dose-response relationship.

DISCUSSION

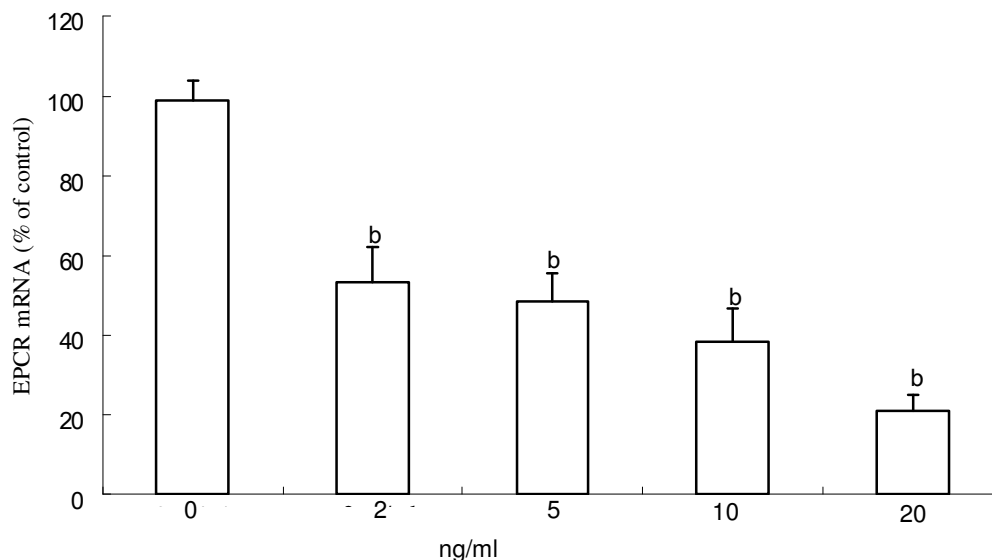EPCR is a type I transmembrane glycoprotein, composed of 238 amino acids, including a signal peptide of 17

Figure 3. Effects of different concentrations of TNF-α on EPCR expression. The mRNA level of EPCR was significantly decreased. A, $p < 0.05$; b, $p < 0.01$, compare with the control (0 ng/ml).
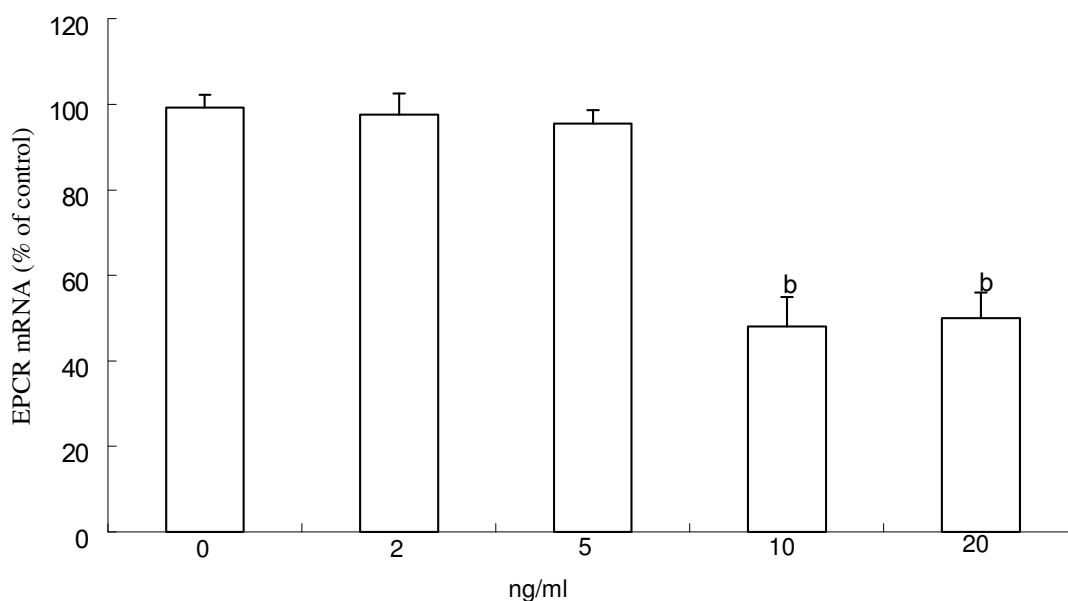

Figure 4. Effects of different concentrations of IL-1β on EPCR mRNA expression. The mRNA level of EPCR was significantly decreased. a, $p < 0.05$; b, $p < 0.01$, compare with the control (0 ng/ml).

amino acids, 46 kD molecular weight. EPCR has several functional domains, including N terminal signal peptide sequences, α1 and α2 extracellular domain, transmembrane domain and a short cytoplasmic tail. The gene encoding EPCR is located in the long arm of chromosome 20, full-length EPCR gene is 6 kb, including 4 exons and 3 introns. EPCR is not only expressed in vascular endothelial cells, but also in the renal cortex, bone marrow, lymph nodes and adrenal glands, as observed by immunohistochemistry detection (Fukudome et al., 1994; Fukudome et al., 1996).

EPCR has two main functions, involving an anticoagulant response and an anti-inflammatory response of the body. When pathological factors activate the coagulation system, prothrombin is activated into thrombin, forming a complex with thrombomodulin in the endothelial cell surface and activating protein C. Activated protein C combines with endothelial cell surface
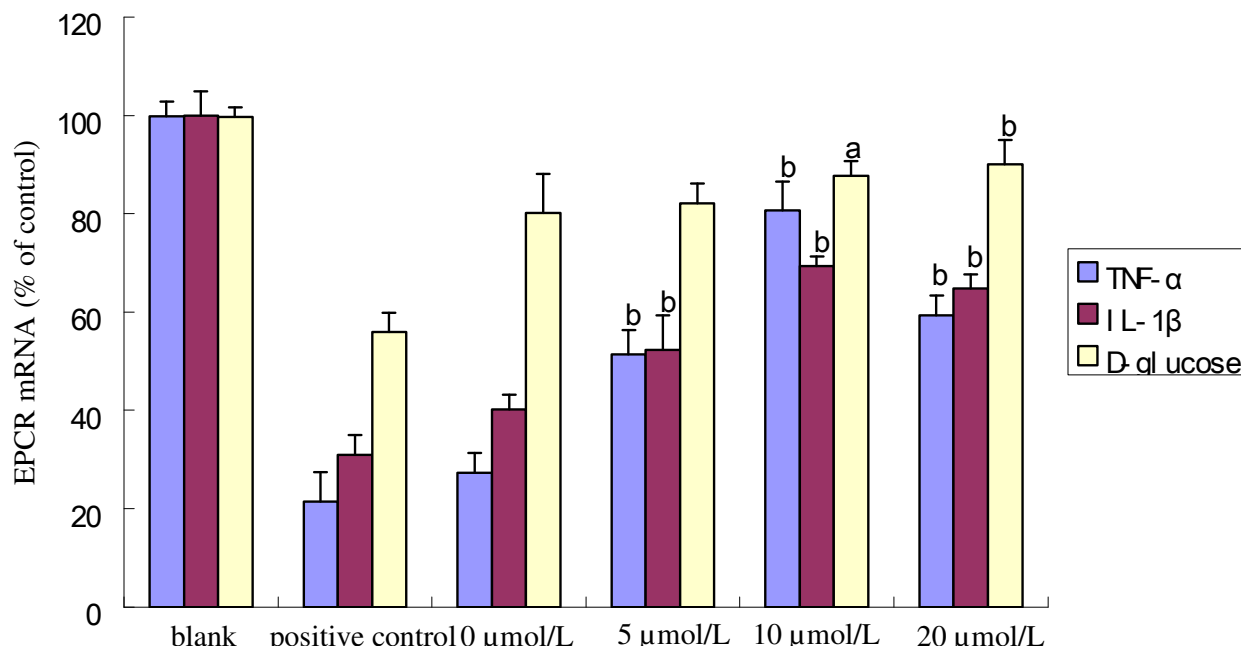
Figure 5. Effects of different concentrations of troglitazone on EPCR expression of RTECs stimulated with high glucose, TNF-α and IL-1β. Troglitazone could significantly improve the inhibition of high glucose, TNF-α and IL-1β. a, $p < 0.05$; b, $p < 0.01$, compare with the control (0 µmol/L).

EPCR, thus further activation; the presence of protein S, prevents further thrombus formation and starts the fibrinolytic system. Some studies show that, APC activity can increase 20-fold when combined with EPCR. Moreover, APC has the effect of an anti-inflammatory response and EPCR is also involved in the process (Ye et al., 1999; Isermann et al., 2005; Taylor et al., 2000; Riewald et al., 2002). APC has a protective effect on endothelial injury in inflammation, can act directly on endothelial cells and down regulating the expression of two subunits (P50 and P52) of NFB. Thus, the expression of TNF of downstream genes, as well as expressions of adhesion molecule ICAM1, VCAM1, and E selection. APC also increases expressions of anti-apoptotic genes such as Bcl2 in endothelial cells (Cheng et al., 2003; Crawley et al., 2002). The study found that, EPCR and human histocompatibility complex ClassI/CD1 molecules showed a high homology, suggesting that it may be involved in the regulation of inflammation response (Simmonds et al., 1999; Shua et al., 2000; Blazs et al., 2006). Clinical studies confirm that, protein C injection is of use in the treatment of meningococcal sepsis and septic shock. This may be related to the inhibition of NFκB caused by APC/EPCR (Saposnik et al., 2004; Stearns et al., 2002).

Studies on EPCR can provide insight into the change processes in the coagulation system and anti-inflammatory mechanism under pathological conditions. The RTECs cell line was chosen for the object of this study, because it shows high EPCR expression in RTECs; further confirming the EPCR gene expression in RTECs by RT-PCR and gene sequencing. In the experiment, we further examined the effects of high glucose, TNF-α and IL-1β on EPCR expression of RTECs and also observed whether troglitazone has an antagonistic effect on the influencing factors. In our study, the choice of the concentration of reagents, such as high glucose, was based on the premise that it does not affect the appreciation of endothelial cells or its adhesion molecule levels. The observation of RTECs culture under stimulation of high glucose shows that, high glucose can lower the EPCR expression in RTECs. However, after the pretreatment of troglitazone to cells, the inhibitory effect of high glucose was partially mitigated. It indicates that the inhibition of EPCR expression is one of the specific ways in which high glucose damages endothelial cells.

TNF-α is a pro-inflammatory cytokine, the key to initiating an inflammatory response and the main factor leading to endothelial cell injury (Lam et al., 2000; Louis et al., 1998). In particular, RTECs injury caused by TNF-α is one of the pathogeneses of interstitial nephritis. In addition, IL-1β is also an important inflammatory cytokine, which induces endothelial cell injury, participates in immune regulation and mediates inflammatory response (Jung et al., 2003). Under certain conditions, IL-1 and TNF-α show mutual regulation. IL-1 can stimulate synthesis and secretion of other cytokines such as TNF-α, IL-6, IL-8 and platelet-derived growth factor and collaboratively produce biological effects, which leads to tissue damage (Esmon et al., 1999; Hata et al., 2004). In

our study, TNF-α and IL-1β can both reduce EPCR expression.

Troglitazone is a thiazolidinedione drug (Kellerer et al., 2003). Such a drug is a new type of insulin sensitizer being used in clinical practice in recent years. Not only can it improve insulin resistance and lower blood sugar, but it can also play a role in non-antihyperglycemic ways such as directly blocking TNF-α-mediated endothelial cell apoptosis, caspase-3 activation, Bcl-2 expression and lipid peroxidation (Tsuchiya et al., 2003; Gouni-Berthold et al., 2001). Thus, troglitazone can alleviate endothelial damage, improve damaged endothelial function, inhibit inflammatory response and protect endothelial function. Our study found that, troglitazone significantly improved the inhibition of glucose and inflammatory factors to EPCR, suggesting that it improves endothelial function by antihyperglycemic and anti-inflammatory ways to play a therapeutic effect.

RTECs injury is the pathological basis of renal interstitial fibrosis and chronic renal insufficiency and its severity indicates the prognosis of chronic kidney disease. Therefore, studies on the detection indicators of damage, its pathogenesis and treatment are both emphasized clinically. It can be seen from our observations on RTECs expression that, EPCR gene reduces by some factors. However, troglitazone can alleviate such effects, such as protecting endothelial cells and resisting inflammation by improving EPCR expression.

Whether or not there is a protective effect of troglitazone on EPCR *in vivo* remains to be further observed. In conclusion, from this study, we have a better understanding of the changes in EPCR in the endothelial and in renal tubular epithelial injury and the protective effect of troglitazone. The result of this study provides a new clue for clinical observation and treatment of diseases such as interstitial nephritis.

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