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Downregulation of endothelin A receptor (ETaR) ameliorates renal ischemia reperfusion injury by increasing nitric oxide production

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ABSTRACT

Aims: To investigate the protective effects of downregulating ETaR expression on renal ischemia reperfusion injury (IRI).

Main methods: The renal IRI model was generated by clamping the left renal artery for 60 min followed by nephrectomy of the right kidney. ETaR siRNA were perfused through the renal artery during ischemia. HE staining was performed to assess histological injury. PCR was performed to determine the expression of NF-κb, TNF-α, IFN-γ, IL-6 and TGF-β. ELISA was used to determine the levels of ET-1, TGF-β and eNOS. The level of nitric oxide (NO) was tested by the NO detection kit. The expression of PI3K, Akt, sGC and PKG were evaluated by western blot.

Key findings: ETaR siRNA treatment reduced the levels of serum creatinine and urea nitrogen, decreased the number of apoptotic cells, and ameliorated histological damage after IRI. PCR results demonstrated that IRI increased mRNA levels of inflammatory factors, which were inhibited by ETaR siRNA treatment. ELISA result showed that ETaR siRNA decreased the levels of ET-1, TGF-β and eNOS in the renal tissues after IRI. Western blot results demonstrated that ETaR siRNA activated the PI3K/Akt and sGC/PKG signaling pathway. Conversely, the NOS inhibitor, L-NAME, reversed the effects of ETaR siRNA treatment.

Significance: ETaR siRNA treatment inhibited inflammatory response and improved renal function after renal IRI. The underlying mechanisms of ETaR siRNA treatment may be through increasing eNOS activity through PI3K/Akt signaling, which subsequently increased NO production. The increased NO reduces the expression of ET-1 by inhibiting transcription of ET-1-associated genes via the sGC/PKG signaling pathway.

1. Introduction

Renal transplantation is currently the most effective treatment for end-stage renal disease. During transplantation, ischemia reperfusion (IR) is an inevitable outcome. One of the major causes for postoperative renal allograft complications is IRI, which leads to delayed graft function, acute kidney injury and graft rejection [1,2]. The pathophysiological changes induced by IRI include cell apoptosis, hemodynamic changes, oxidative stress and inflammatory response [3]. Previous studies have shown that inhibiting excessive constriction of renal blood vessels and injury to vascular endothelial cells during early IR ameliorates IRI to the transplanted kidney [4].

Endothelin (ET) is a strong endogenous vasoconstrictor, and is classified into three subtypes, ET-1, ET-2 and ET-3. ET-1 is mainly produced by endothelial cells and is up-regulated significantly after renal IRI [5]. The two key types of ETR include ETaR and ETbR. The ETaR is mainly found on the surface of vascular smooth muscle cells, while the majority of ETbR is found on the surface of endothelial cells [6]. When ET-1 binds to ETaR on vascular smooth muscle cells, it induces vasoconstriction and vasospasm and further aggravates renal ischemia. However, ET-1 binding to ETbR induces NO and prostaglandin production and leads to vasodilation and renal ischemia amelioration [6]. NO is a non-classical neurotransmitter produced by nitric oxide synthase (NOS) catalyzing L-arginine. Depending on the

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source, NOS is divided into neural (nNOS), endothelial (eNOS) and induced (iNOS). The nNOS is mainly found in the nervous system. However, it is also found in macula densa, proximal tubule, distal tubule and collecting ducts [7]. The eNOS is primarily in renal tubular epithelial cells and vascular endothelium of the kidneys [8–10]. The iNOS is mainly found in macrophages. It participates in inflammatory response, the up-regulation of which leads to renal injury [11]. Whereas eNOS is involved in prevention of renal vasospasm and reduction of inflammatory cell infiltration [23]. The mechanisms regulating NO synthesis in the kidney are similar to those in other tissues. It is reported that vascular endothelial growth factor (VEGF) in glomerular endothelial cells stimulates NO synthesis via activation of the PI3K/Akt pathway leading to activating phosphorylation of eNOS-Ser^{1177} [12]. The impairment of NO synthesis may lead to reduced renal blood flow, increased renal vascular resistance and ET-1 expression. On the other hand, high level of ET-1 impairs endothelial NO production via PKC-dependent pathway and lack of ET-1 not only attenuated proximal tubular injury in response to IR, but also decreased inflammatory and oxidative stress responses [13,14]. Therefore, increasing of NO is capable of repressing local ET-1 expression through feedback inhibition.

NO acts on smooth muscle cells to induce vasodilation by increasing production of the secondary messenger cyclic guanosinc monophosphate (cGMP) via activation of soluble guanylate cyclase (sGC), which is the only known receptor for NO [15,16]. Cyclic GMP-dependent protein kinase (PKG) is a key mediator of the NO/cGMP signaling pathway and plays a central role in regulating cardiovascular functions [17]. However, the mechanisms by which elevated levels of NO reduced ET-1 production have yet to be determined. It is reported that the activity of HIF-1α, AP-1 and NF-κB were increased under chronic hypoxia, causing up-regulation of iNOS and ET-1, which play a significant role in the hepatic response to oxidative stress [18].

RNA interference has been widely used in a variety of organisms to induce specific post-transcriptional gene silencing [19,20]. siRNA is a class of double-stranded RNA molecules that can decrease specific gene expression to modulate biological function. It is demonstrated that inhibition of ETaR mitigated renal structural injury and improved renal function in IRI rats [21]. However, whether exogenous ETaR siRNA could decrease ET-1 expression and increase local NO levels to exert renoprotective effects in rats is yet to be deciphered. In our present study, an IRI rat model was established to determine the underlying mechanisms by which ETaR siRNA offers renoprotection in renal IRI. We found that: (1) ETaR siRNA decreased renal dysfunction and histologic damage by increasing endothelial production of NO mainly through the PI3K/Akt pathway. (2) Inhibition of NOS resulted in the up-regulation of inflammatory factors and aggravated renal IRI by suppressing the sGC/cGMP/PKG pathway.

2. Material and methods

2.1. Animal model

Male Sprague–Dawley rats (200-250 g, 3–4 months) were purchased from Shanghai Slake Experimental Animal Co. Ltd. All animals were kept in cages in the animal house of Zhongshan Hospital affiliated to Fudan University, with free access to food and water. Rats were environmentally adapted for 1 week before initiation of studies. All procedures were approved by the Bioethics Committee of Zhongshan Hospital affiliated to Fudan University.

2.2. Model of unilateral renal IRI establishment

Male Sprague–Dawley rats (200-250 g) were randomly divided into five groups, i.e.,(1) sham group: right kidney nephrectomy was performed; (2) IR group: Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg body weight). The left kidney was clamped for 60 min followed by resection of the right kidney. After a medial abdominal incision, the left renal artery was clamped for 60 min using a serrefine. Adequate restoration of blood flow after clamp removal was verified before abdominal closure. The right kidney was then removed and the incision was sutured using 3–0 silk tread; (3) Negative siRNA group: IR rats were administered through the renal artery 30 min before renal clamping; (4) ETaR siRNA group: IR rats were administered with ETaR siRNA; (5) To investigate the silencing effect and protective efficacy of ETaR siRNA on the renal tissue of I/R injury, the rats were administered with ETaR siRNA during ischemia. ETaR siRNA plasmid DNA (100 μg) and L-NAME (20 mg/kg) were diluted in phosphate-buffered saline (PBS) and perfused into the kidney through the renal artery. At 48 h post-ischemia, the survival rate in the sham, IR, Negative siRNA, IR + ETAR siRNA, IR + ETaR siRNA + L-NAME groups were 10/10, 6/10, 9/10, 8/10 and 6/10 respectively. The sequence of ETaR siRNA: 5′-CACGACGGCUUUCAAAUAUTT-3′ and 5′-AUAUUUGAAAGCCGUCGUGTT-3′, the sequence of negative siRNA: 5′-UUCUCCGAACGUGUCACGUTT-3′ and 5′-ACGUGACACGUUCGGAGAATT-3′.

2.3. Renal function

Blood samples were obtained from the inferior vena cava and centrifuged at 3500 r/min for 10 min at 4 °C. After that the supernatant serum was collected and levels of serum creatinine and urea nitrogen was measured by the core laboratory at the SLAC Laboratory Animal Center to assess the renal function. Afterwards, the renal function for each group was analyzed.

2.4. Histopathology and cell apoptosis

Renal tissues were embedded in paraffin following fixation with 10% formalin, and then subsequently cut into about 5 μm thick slices. After dewaxing, HE and TUNEL staining was performed. HE staining was semi-quantitatively graded under microscopy at 200× to evaluate tissue damage. The grader of the histology slides was examined blindly by a pathologist. The percentage of histological changes in the cortex and medulla was scored using a semi-quantitative scale designed to evaluate the degree of the necrosis in tubular and glomerular areas, tubular vacuolization and cast formation on a five point scale based on injury area of involvement as previous study [22]. The scale is as follows: 0 ≤10%; 1 = 10–25%; 2 = 25–50%; 3 = 50–75%; and 4 = 75–100%. Cell apoptosis was detected using the apoptosis test kit (Millipore, MA, USA). Apoptotic cells were semi-quantitated using 20 fields of view at 200× magnification.

2.5. Quantitative real-time polymerase chain reaction

Total RNA was extracted from renal tissues using Trizol reagent (Invitrogen, Carlsbad, USA). 1 μg of total RNA was then reverse transcribed into cDNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, USA). Real-time quantitative PCR (qPCR) was performed using SYBR Premix Ex Taq Kit (Takara Bio Inc., Otsu, Japan). After a hot start (30 s at 95 °C), amplification was performed for 40 cycles (5 s at 95 °C, 30 s at 55 °C, 60 s at 72 °C). Primers used for qRT-PCR are listed in Table 1. Expression levels were normalized to GAPDH using the $2^{-\Delta\Delta Ct}$ method.

2.6. ELISA

Renal tissues were grinded in lysate and centrifuged for 20 min at 2000 r/min at 4 °C. Supernatant or standard sample was added to the ELISA plate according to the Xitang Biotech Elisa kit protocol. After brief mixing, the plate was incubated at 37 °C for 40 min and then primary antibody working solution, enzyme labeled antibody and substrate working solution was added sequentially after washing the plate. OD values were measured at 450 nm wavelength after adding

Table 1
The sequence of gene-specific primers.

	The sequence of gene-specific primer
ETaR	F-ACATGCTGGCTGGTTCTGGAG- R-ATGGGCAGGAGTGTAGCCAGTC-
NF-κb	F-CACTTAGCCATCATCCACCTTC- R-AGTCCTCCACCACATCTTCC
TGF-β	F-CTTGCCCTCTACAACCAACA R-CTTGCGACCCACGTAGTAGA
IL-6	F-CCACTTCACAAGTCGGAGGCTTA R-GTGCATCATCGCTGTTCATACAATC
ET-1	F-ACCTGGACATCATCTGGGTCAAC R-TTTGGTGAGCACACTGGCATC
IFN-γ	F-ATGGATGCTATGGAAGGAAAGAG R-CACTTATGTTGTTGCTGATGGC

stop solution. The relative sample content was calculated using the standard concentration gradient as abscissa and OD value as ordinate.

2.7. Western blot

Renal tissues were homogenized in ice-cold radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Nantong, China). Lysates were then centrifuged at 12,000 × *g* for 25 min at 4 °C, following which the supernatant was collected and total protein were quantified using the bicinchoninic acid assay (Beyotime Institute of Biotechnology, Haimen, China) per manufacturer's protocol. 20 mg protein from each sample was then separated by SDS-polyacrylamide gel and transferred to polyvinylidene fluoride membrane at 300 mA for 120 min. Primary antibodies were incubated at 4 °C overnight, i.e., ETaR(ab85163, abcam, US), PI3K(#4228, Cell Signaling, USA), p-Akt(#4060, Cell Signaling, USA), sGC(ab14438, abcam, US), and PKG(#3248, Cell Signaling, USA).Relative protein expression was subsequently quantitated by normalizing to β-Actin (#3700, Cell Signaling, USA) levels using the Image-Pro plus 6.0 software.

2.8. NO detection

The concentration of NO in renal tissue was determined by commercially available NO detection kit (Beyotime Institute of Biotechnology, Nantong, China). Renal tissue lysate was centrifuged and supernatant was collected. The protein concentration in the supernatant was determined using a Pierce BCA protein assay kit (Thermo Scientific). Total NO production in kidney tissues was determined by measuring the concentration of nitrate and nitrite, a stable metabolite of NO, using a modified Griess reaction method following the protocol provided with a Total NO Assay Kit.

2.9. Data analysis

Data was expressed as mean ± standard deviation (SD), and statistical analysis was performed using Students *t*-test or One-way analysis of variance (ANOVA) with post hoc contrasts by Student-Newman-

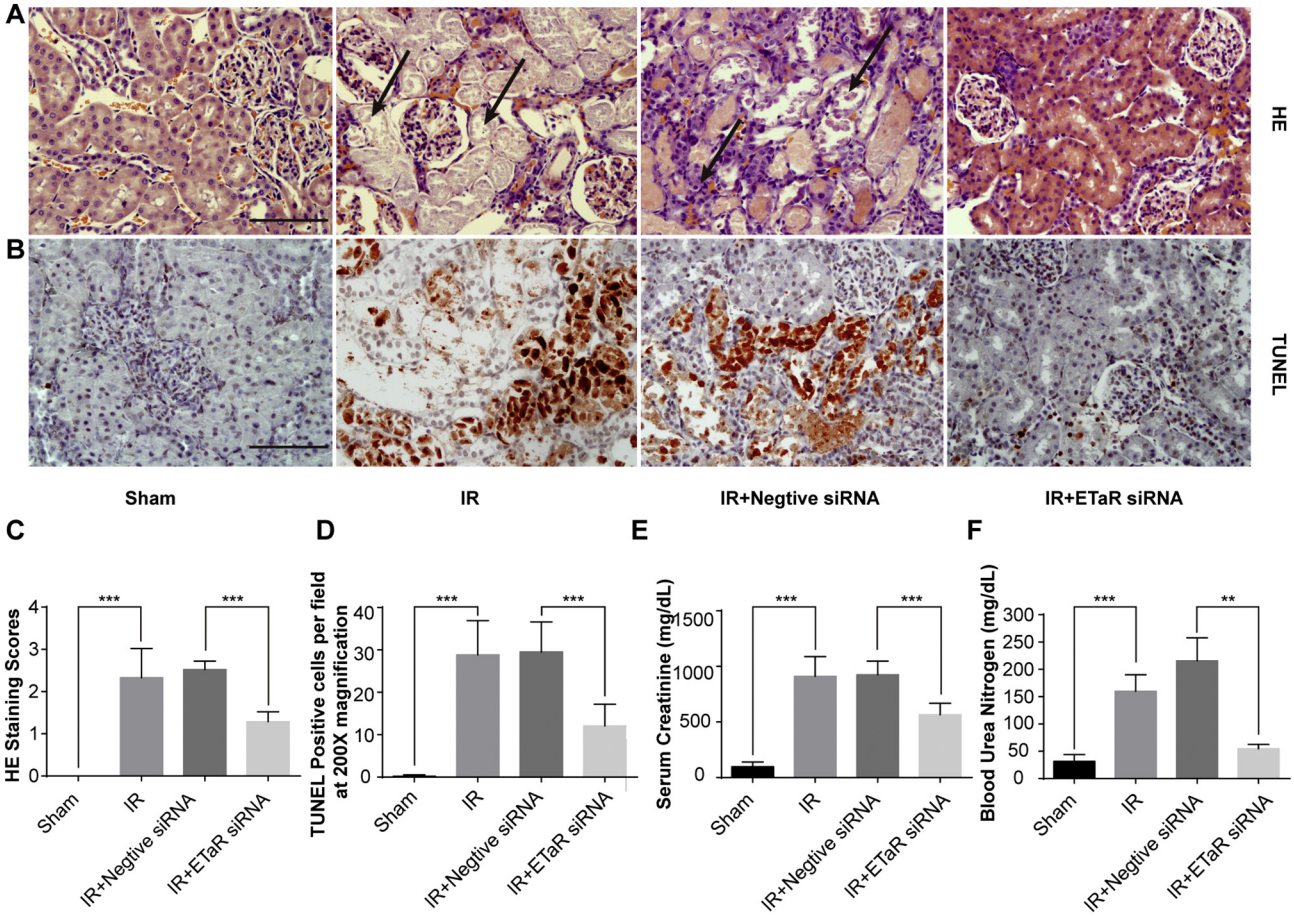

Fig. 1. Renoprotective function of ETaR siRNA after renal IRI. (A, B) Representative images of HE and TUNEL staining in rat kidneys with or without ETaR siRNA administration after IR. Images were captured at magnification, × 200, scale bar = 10 μm. Arrows indicate pathological changes including inflammatory cell infiltration, dilated tubules, interstitial expansion. (C, D) shows summarized group results for HE staining scores and the number of TUNEL positive cells. Histopathological grading of tissue injury was assessed using the 0- to 4-point scoring system. (E, F) Serum creatinine and urea nitrogen levels were measured using the peripheral blood automatic biochemical analyzer 48 h after IR. Data are expressed as mean ± SD, $^{**}P < .01$, $^{***}P < .001$, $n = 6$.

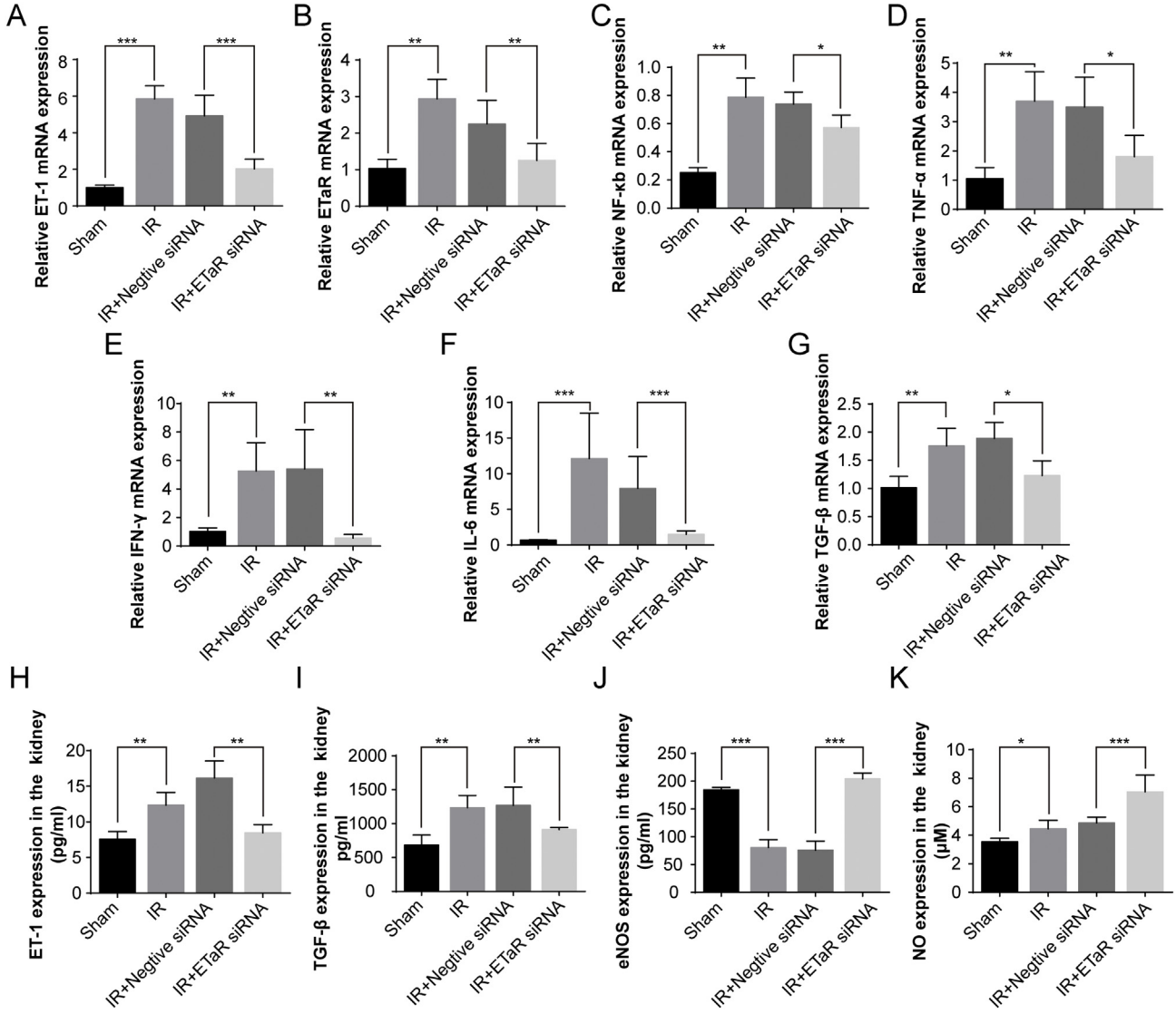

Fig. 2. ETaR siRNA decreased inflammatory and associated transcription factors in IRI rats. (A–G) Total RNA was extracted from renal tissue 48 h after IR, then reversed transcribed into cDNA. ETaR siRNA administration reduced the mRNA levels of ETaR, ET-1, NF-κb, TNF-α, IFN-γ, IL-6 and TGF-β while negative siRNA had no effect. (H–K) Renal tissues were collected 48 h after IR and related indexes were measured by ELISA. (J) The levels of NO was detected by commercially available NO detection kit. ETaR siRNA administration decreased the levels of ET-1and TGF-β, and increased eNOS and NO levels. However, the negative siRNA group had no significant differences. Data was expressed as mean ± SD, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $n = 6$.

Keuls test. A P value < .05 was considered statistically significant.

3. Results

3.1. ETaR siRNA reduced renal tissue damage, cell apoptosis and improved renal function induced by IR

HE and TUNEL staining were performed to assess the degree of renal injury and tissue apoptosis for each treatment group. The 0- to 4-point scoring system was used to evaluate tissue injury. The result demonstrated that rats undergoing renal IRI had tissue injury, increased inflammatory cell infiltration and larger areas of tubular necrosis, vacuolization and cast formation. Rats pretreated with ETaR siRNA had significant attenuation of pathological damage to the kidney, while negative siRNA had no significant effect (Fig. 1A, C). In addition, TUNEL staining demonstrated that cell apoptosis increased after IRI. ETaR siRNA treatment significant decreased the number of apoptotic cells, while no significant decrease of renal cell apoptosis was observed in the negative siRNA group (Fig. 1B, D). This result demonstrated that ETaR siRNA had a strong protective role on kidney damage in IRI rats. Blood and renal tissue samples were collected 48 h after IR. Serum creatinine and urea nitrogen levels were measured using the peripheral blood automatic biochemical analyzer. The levels of serum creatinine and urea nitrogen increased significantly 48 h after renal IR, however after ETaR siRNA administration, renal function indexes decreased significantly, while administration of negative siRNA did not cause significant differences in renal function induced by IR (Fig. 1E, F).

3.2. ETaR siRNA down-regulated inflammatory factors and increased NO production in the kidneys of IRI rats

To investigate whether ETaR siRNA has the anti-inflammatory effect in renal IRI, we further examined the mRNA level of inflammatory factors in rat kidney tissues. Results showed that mRNA levels of NF-κb, TNF-α, IFN-γ, IL-6 and TGF-β were significantly increased 48 h after renal IR and were reduced by ETaR siRNA (Fig. 2A–G). In addition, the levels of ET-1, eNOS, TGF-β and NO level in renal tissues were measured by ELISA and NO detection kit. The levels of ET-1, NO and TGF-β

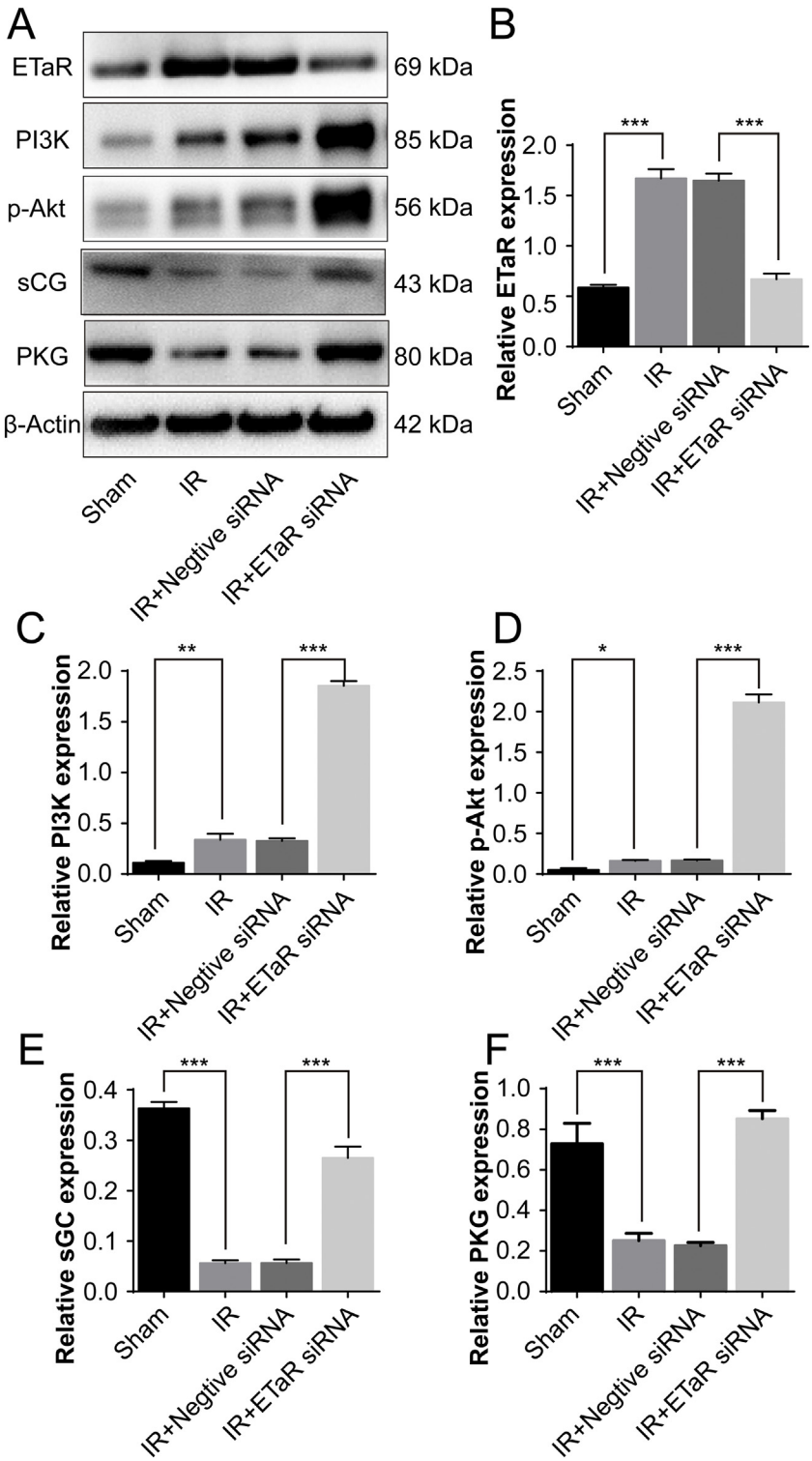

Fig. 3. ETaR siRNA activates PI3K/Akt and sGC/cGMP/PKG signaling pathways after IR. Proteins were extracted from the kidneys from each group 48 h after IR surgery. (A–F) Representative images and summarized data showing the protein levels of ETaR, PI3K, p-Akt, sGC and PKG. The expression levels of ETaR, PI3K, p-Akt, sGC and PKG were normalized to β-actin levels within the same sample. Data were expressed as mean ± SD, *P < .05, **P < .01, ***P < .001, $n = 6$.

were significantly increased, while eNOS levels were decreased in the IR group. Administration of ETaR siRNA reduced ET-1 and TGF-β levels and increased eNOS and NO levels (Fig. 2H–K). As previous study demonstrated that NO plays critical role in renal IRI [23], the result suggested that ETaR siRNA may have a protective role in IRI through the eNOS-related signaling pathway.

3.3. ETaR siRNA activated PI3K/Akt and sGC/cGMP/PKG signaling pathways in the kidneys after IR

The PI3K/Akt and sGC/cGMP/PKG signaling pathways were analyzed using Western Blot. ETaR expression was upregulated after IR, but was decreased significantly after ETaR siRNA administration (Fig. 3A, B). 48 h after IR, PI3K and p-Akt expression levels in renal tissues were

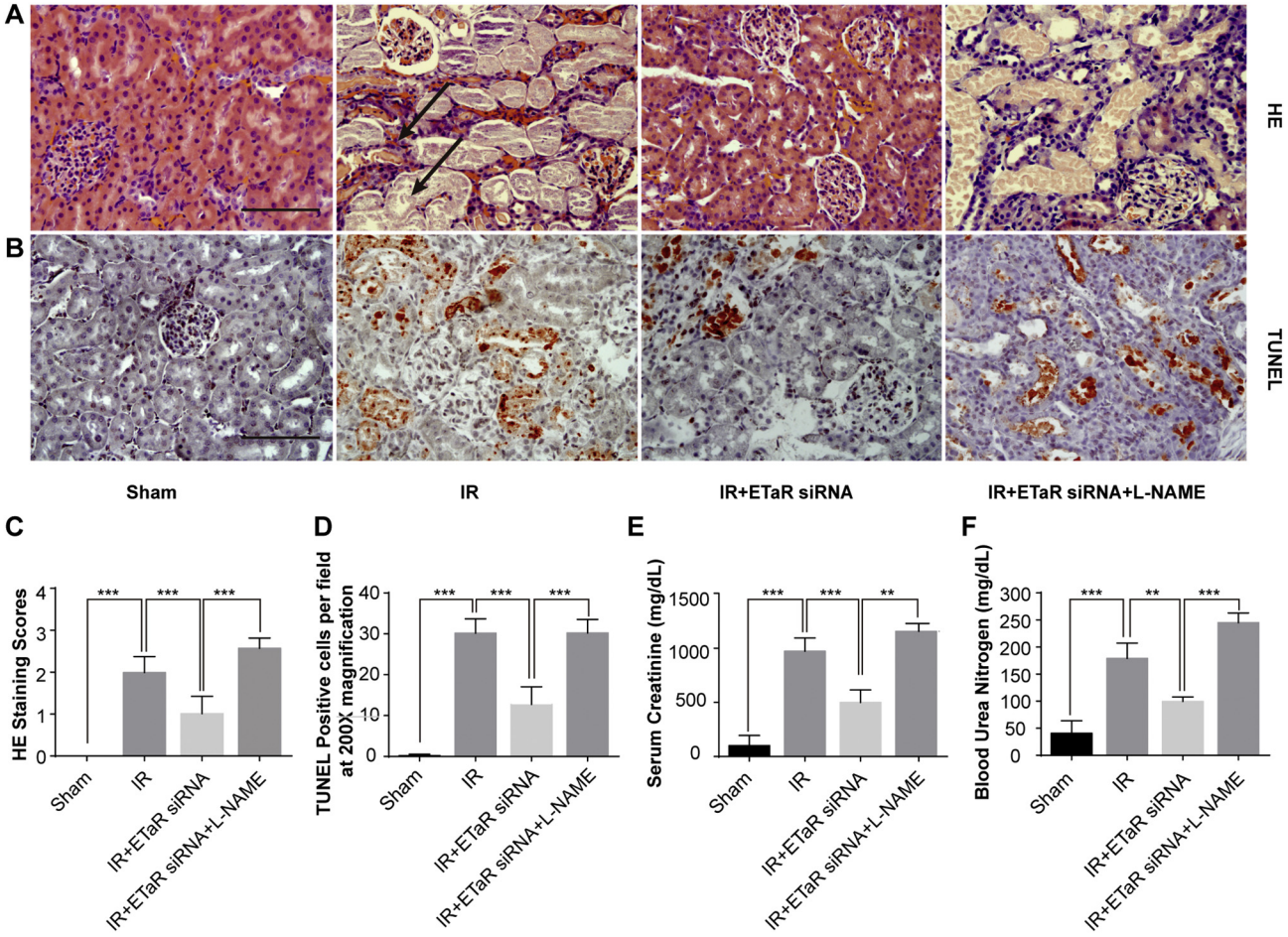

Fig. 4. L-NAME reversed the renoprotection effects of ETaR siRNA administration. (A, B) Representative images of HE and TUNEL staining in rat kidneys with or without administration of ETaR siRNA or L-NAME. Images were captured at magnification, ×200, scale bar = 10 μm. Arrows indicate pathological changes including inflammatory cell infiltration, dilated tubules, interstitial expansion. (C, D) Group data summarizes the results for HE staining scores and the number of TUNEL positive cells. Histopathological grading of tissue injury was assessed using the 0- to 4-point scoring system. (E, F) Serum creatinine and urea nitrogen levels were measured using the peripheral blood automatic biochemical analyzer 48 h after IR. Data were expressed as mean ± SD, $^{**}P < .01$, $^{***}P < .001$, $n = 6$.

increased and further increased after ETaR siRNA treatment. The expression levels of sGC and PKG were decreased 48 h after IR but were upregulated after ETaR siRNA treatment (Fig. 3A, C–F).

3.4. The NOS inhibitor, L-NAME, reversed ETaR siRNA-induced renoprotection after IRI

It has been reported that NO inhibits the expression of ET-1 mRNA [24]. Hence, we wondered if downregulation of ETaR could reduce expression of ET-1. To investigate the molecular mechanism of NO in ET-1 regulation, we used L-NAME, an NOS inhibitor. HE staining showed that rats administered L-NAME reversed ETaR siRNA-induced attenuation of pathological damage in rat kidneys. TUNEL staining showed that treatment with ETaR siRNA decreased the number of apoptotic cells, but was increased after L-NAME administration (Fig. 4A–D). We also detected the change of serum creatinine and urea nitrogen levels after L-NAME treatment. Our results showed that serum creatinine and urea nitrogen levels were reduced significantly in the ETaR siRNA group, while L-NAME treatment reversed this effect (Fig. 4E, F).

3.5. Effect of L-NAME on the PI3K/P-Akt and sGC/cGMP/PKG signaling pathway

The signaling pathways were analyzed by Western blot after L-NAME administration. The PI3K and P-Akt expression levels in the ETaR siRNA group were higher compared to the IR group, while L-NAME administration decreased the levels of P-Akt but had no effect on PI3K expression (Fig. 5A, B, C). L-NAME had no significant effect on ETaR expression (Fig. A, D). PKG and sGC expression levels were reduced after L-NAME compared to the ETaR siRNA group (Fig. 5A, E, F). This suggests that L-NAME acts downstream rather than upstream of the NOS enzymes pathway. Thereby inhibition of NOS leads to decreased production of NO, resulting in the inactivation of the sGC/cGMP/PKG signaling pathway to increase expression of ET-1.

3.6. L-NAME reversed the inhibitory effect of ETaR siRNA on inflammatory factors

To investigate whether L-NAME could affect the mRNA levels of inflammatory cytokines and transcription factors in rat kidneys, we measured the mRNA levels of inflammatory cytokines and transcription factors after L-NAME pretreatment. We found that the mRNA expression of NF-κb, TNF-α, IFN-γ, IL-6 and TGF-β were significantly increased after L-NAME administration compared to the ETaR siRNA group (Fig. 6A–G). L-NAME reversed ETaR siRNA-induced down-regulation of ET-1 and TGF-β and up-regulation of NO in renal tissues (Fig. 6H–J). These results indicate that NO may be upstream and participates in proinflammatory response and ET-1 target gene transcription.

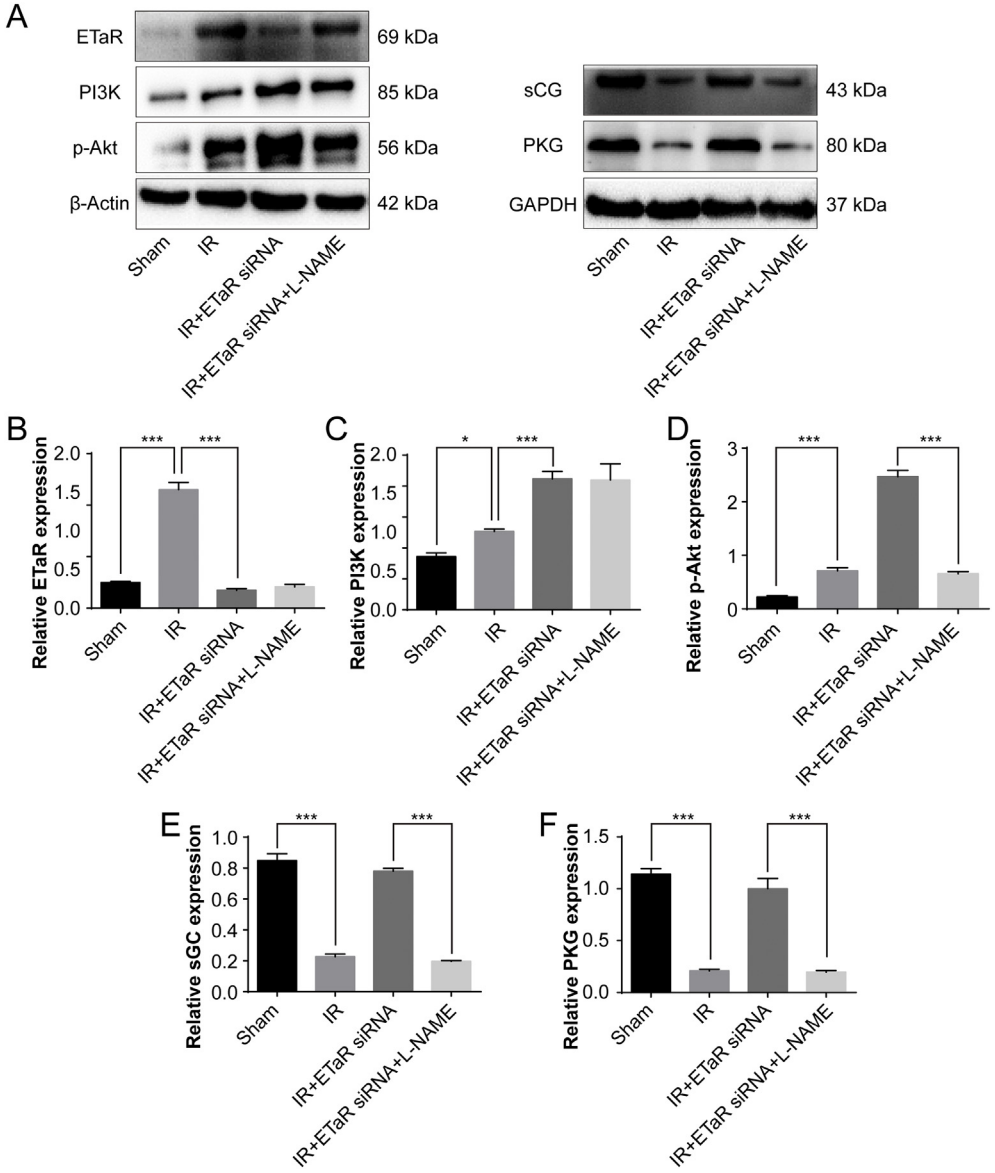

Fig. 5. L-NAME inhibits ETaR siRNA-induced activation of the sGC/cGMP/PKG signaling pathway. (A–F) Representative images and summarized data showing the protein levels of ETaR, PI3K, p-Akt, sGC and PKG. Expression of ETaR, PI3K, p-Akt, sGC and PKG were normalized to β-actin levels in the same sample. Data were expressed as mean ± SD, *$P < .05$, ***$P < .001$, n = 6.

4. Discussion

IRI is an unavoidable problem during renal transplantation, and causes short- and long-term post-transplant renal dysfunction [25]. Preventing IRI after renal transplantation is essential for renal function recovery and long-term graft survival. Endothelin system disorders are the key pathophysiological features of renal IRI. Regulation of the ET system has been reported to sufficiently control kidney graft injury [26]. In our study, we investigated the protective effects of decreasing ETaR expression by ETaR si RNA on renal IRI in a rat IR model.

We found that kidneys of rats with renal IRI had extensive tissue injury, inflammatory cell infiltration, large area of tubular necrosis, vacuolization and cast formation, while administration of ETaR siRNA ameliorated these symptoms significantly (Fig. 1A–D). The serum creatinine and urea nitrogen levels increased significantly 48 h after renal IR, whereas ETaR siRNA administration decreased renal function indexes (Fig. 1E, F). As previous study showed that ETaR antagonist mediates the protective effect via a NO-dependent mechanism [27]. Our result suggested that ETaR siRNA plays a critical role in ameliorating renal IRI. ET-1 is modulated by several factors. It is increased by TNF-α, INF-γ, TGF-β, ANGII production and decreased by NO and prostaglandin [28,29]. To investigate the specific mechanism, we examined the expression of associated pro-inflammatory and transcription factors.

TNF-α induces endothelial cell damage, thereby inducing vascular dysfunction and thrombosis [30,31]. Furthermore, it enhances neutrophil phagocytosis via antibody-dependent cell mediated cytotoxicity (antibody-dependent cell-mediated cytotoxicity, ADCC), promotes neutrophil attachment to vascular endothelial cells, induces oxygen free radical and cell apoptosis. This results in the activation of local inflammatory responses to aggravate renal IRI [32]. Studies have shown that TGF-β signaling is transferred from the cell surface to the nucleus via Smads activation. The activated Smads then translocate to the nucleus and activate the transcription of downstream target genes. This results in augmenting the local inflammatory response [33]. NF-κb is a major regulator of the innate immune response, cell survival and inflammation [34]. The above mentioned nuclear transcription factors play a critical role in ET-1 gene transcription. Thus, we examined their

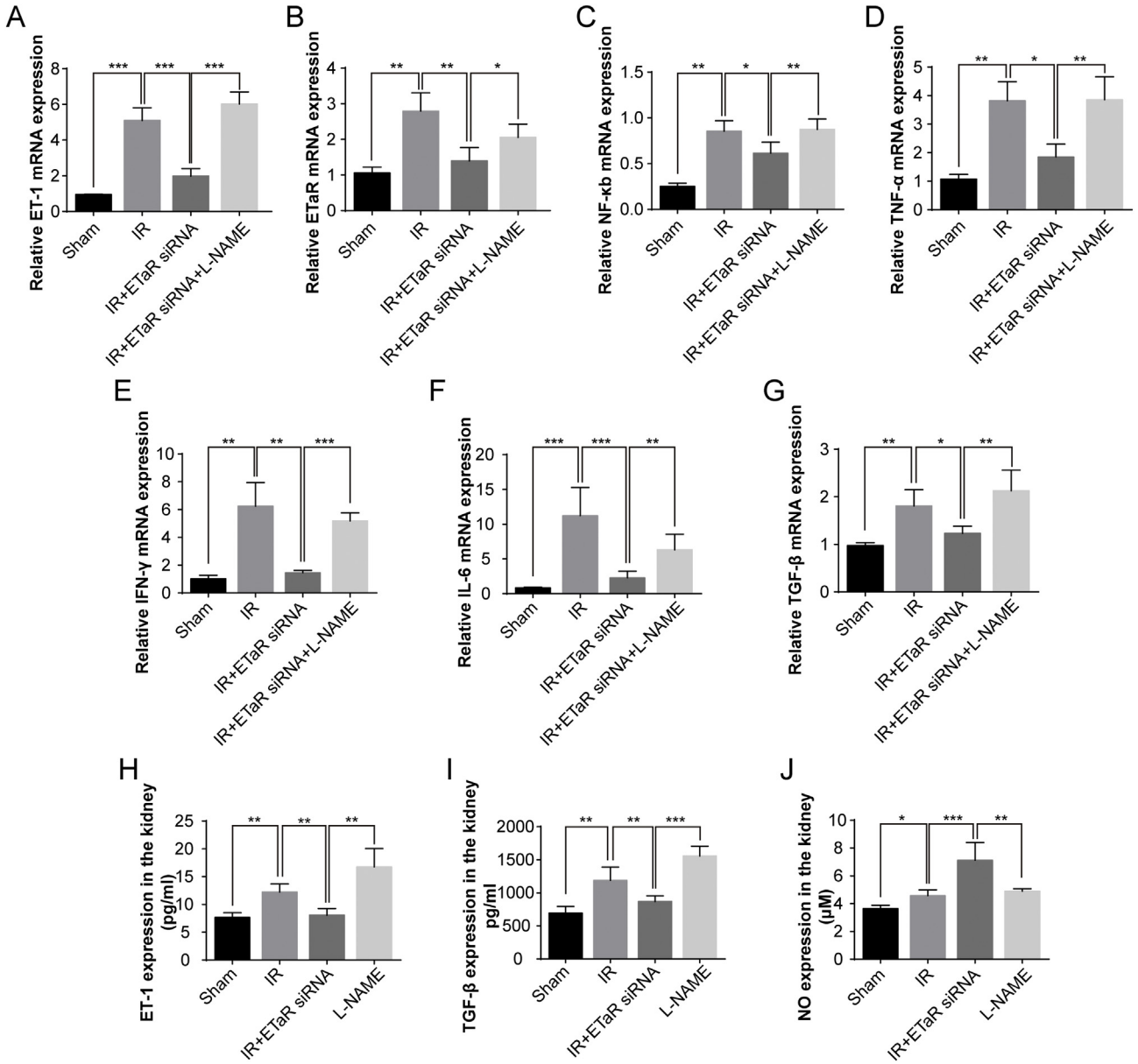

Fig. 6. L-NAME reverses the inhibitory effects of ETaR siRNA on inflammatory and transcription factors (A–F). The mRNA levels of ETaR, ET-1, NF-κb, IFN-γ, IL-6 and TGF-β were reduced after ETaR siRNA administration, but were increased after L-NAME administration. (H–I) Renal tissues were collected 48 h after IR and related indexes were examined by ELISA. (J) The levels of NO was detected by commercially available NO detection kit. ETaR siRNA treatment decreased the levels of ET-1, TGF-β and increased the levels of NO. However, this effect was reversed by L-NAME. Data were expressed as mean ± SD, *P < .05, **P < .01, ***P < .001, n = 6.

expression levels after ETaR siRNA administration. We found that ETaR siRNA treatment decreased mRNA levels of NF-κb, TNF-α, IFN-γ, IL-6 and TGF-β (Fig. 2A–G). In addition, we found ETaR siRNA significantly increased eNOS expression, NO level and decreased ET-1 level in renal tissues (Fig. 2H, J, K). As ET-1 deletion is sufficient to halt pathological changes in tubulointerstitial and renal artery morphology as well as increase in oxidative stress in rats undergoing renal IR [35]. This indicates a link between ETaR siRNA and increased ET-1. ETaR siRNA treatment increased eNOS levels, elevated NO production, and the increased NO may provide renal protection by regulating renal blood flow.

PI3K/Akt signaling pathway is involved in cell proliferation, inflammatory response and chemotaxis. It has been reported that renal preischemia or ischemia reperfusion could stimulate the PI3K/Akt signaling pathway [36]. In our study, we found that expression levels of PI3K and p-Akt were slightly increased after IRI in the kidney of rats, while ETaR siRNA treatment could significantly increase their expression to further activate eNOS (Fig. 3A, C, D). The slightly increased expression of PI3K and p-Akt after IRI may relate to the self-protection mechanism as activation of PI3K/Akt signaling pathway exerts renoprotection against IR [37]. Recent studies have demonstrated that vasorelaxation of renal arteries is modulated via the endothelium-independent sGC/cGMP/PKG signaling pathway under hypoxia conditions [38]. As NO inhibits ET-1 production through the suppression of NF-κb [39] We reasoned that sGC/cGMP/PKG signaling may be associated with the production of ET-1. We found that the expression of sGC and PKG in renal tissues was decreased after IR but ETaR siRNA treatment reversed this trend (Fig. 3A, E, F). We found that ETaR siRNA specifically suppressed the expression of ETaR, which consequently resulted in no binding of ET-1 to ETaR (Fig. 3B). Lack of ETaR results in increased ET-1 binding to ETbR and consequently produces more NO via the activation of PI3K/Akt. ETbR is mainly localized on the surface

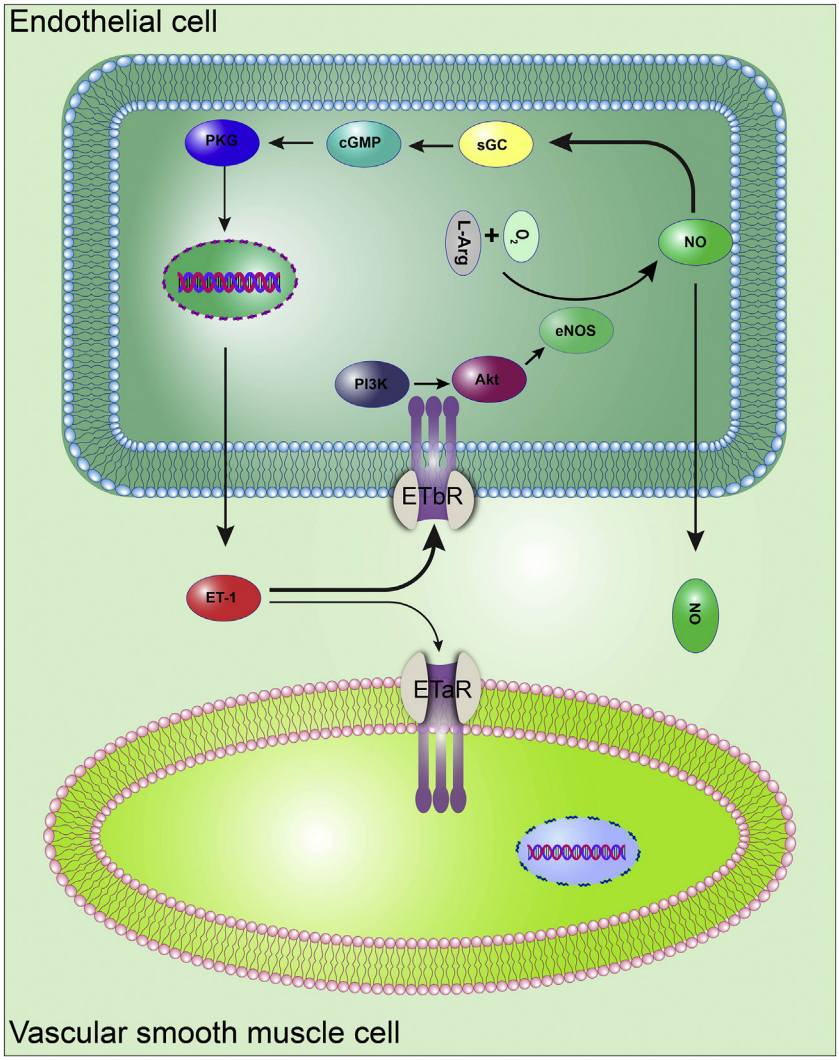

Fig. 7. Model summarizing the effects of ETaR siRNA on renal IRI. ETaR siRNA increased NO levels by activating eNOS through the PI3K/Akt pathway and additionally modulating the sGC/cGMP/PKG signaling pathway to regulate downstream transcription factors.

of endothelial cells. It is documented that ETbR down-regulation is associated with renal IRI [40]. Our study is similar to the previous study that a ETaR antagonist prevents renal IRI in kidney transplantation [21]. Furthermore, blockade of ETA provided dramatic protection of postischemic acute kidney injury (AKI) progression to chronic kidney disease (CKI) [41].

To further investigate whether NO was involved in ET-1 production, an NOS inhibitor, L-NAME was used. We found that L-NAME reversed ETaR siRNA-induced attenuation of pathological damage, apoptotic cells, and serum creatinine and urea nitrogen levels (Fig. 4). This result indicated that ETaR siRNA treatment ameliorated IRI by regulating eNOS-NO system.

NO is produced via the activation of the PI3K/Akt signaling pathway. After L-NAME treatment, the expression of p-Akt was decreased (Fig. 5C). However, expression of PI3K and ETaR did not alter. sGC and PKG levels were reduced after renal IRI and were similar to the L-NAME treated group (Fig. 5). That indicates that L-NAME acts downstream rather than upstream of the eNOS-NO pathway. In subsequent experiments, the effect of L-NAME on ET-1 associated inflammatory and transcription factors were evaluated. ETaR siRNA decreased mRNA levels of NF-κb, TNF-α, IFN-γ, IL-6 and TGF-β and were reversed with L-NAME treatment. In addition, L-NAME increased the production of ET-1 and TGF-β after renal IRI (Fig. 6H, I). The NO levels in renal tissue were increased after IRI, while eNOS levels were significantly reduced. This indicates that increased NO levels after IR was initiated by the up-regulation of iNOS, and results in increased tissue damage (Fig. 2J, K). ETaR siRNA treatment further increased NO and eNOS levels, indicating that reduced ETaR levels upregulated NO via the up-regulation of eNOS. This results in renal blood flow regulation to protect the kidney from damage. However, the NOS inhibitor L-NAME could reverse the protective effects of ETaR siRNA. This result further confirmed that the protective effects of ETaR siRNA were through the regulation of NO levels via the upregulation of eNOS expression.

5. Conclusion

Our results demonstrated that ETaR siRNA upregulates NO via NOS enzymes mainly through the PI3K/Akt pathway. In addition, the production of NO modulates sGC/cGMP/PKG signaling pathway to regulate the activity of ET-1 related transcription factors and then to protect the kidney from IRI (Fig. 7).

Author contribution to study

In this paper, L.L. X.W. and L.Z. carried out the molecular biology studies and the immunoassays, analyzed the data and drafted the manuscript. J.L. M.X. R.R. and T.Z. performed the cell experiments. Y.J.

designed and supervised the study, revised the manuscript and gave final approval for publication.

Declaration of interest

The authors declare that they have no competing interests.

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