Original Paper

Endothelin Receptor Down-Regulation Mediated Ligand Regulation Mechanisms Protect Against Cellular Hypoxia Injury in Rat Vascular Endothelial Cells

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Key Words

Endothelin receptor • Small interfering RNA • Endothelial cells • Co-culture

Abstract

Objective: Investigation of the effect of endothelin receptor A (ETaR)-targeting small interfering RNA (siRNA) on rat vascular endothelial cellular hypoxia injury, as well as its underlying mechanism. **Methods:** An *in vitro* rat vascular smooth muscle cells - endothelial cells co-culture model was established and transfected with ETaR siRNA before hypoxia treatment. Cell culture supernatant, cellular protein and RNA were collected and examined at 0.5hrs, 1hrs, 2hrs, 4hrs, 8hrs, 16hrs, 24hrs and 48hrs of hypoxia with 1% oxygen. The time point at which the best silencing effect was achieved was chosen, eNOS inhibitor L-NAME was added, and post hypoxia cell culture supernatant, cellular protein and RNA was collected for further examination. **Results:** After hypoxic treatment, endothelial-1 (ET-1) and ETaR expression levels gradually increased as oxygen deprivation extended. ET-1 and ETaR expression levels were significantly lower in the ETaR siRNA group compared with the Hypoxia group ($P < 0.001$). Such difference peaked at 4hrs of hypoxia. ELISA examination of cell culture supernatant revealed that the amount of ET-1 and TGF- β in the ETaR siRNA group were significantly lower compared to the Hypoxia group at all times, while the amount of NO and eNOS was higher. After 4 hrs of hypoxia, Smad2, Smad3, HIF-1, TNF- α , IFN- γ , IL-6, MCP-1, NF- κ B, ET-1 and ANG II mRNA expression in endothelial cells and ETaR mRNA expression in A-10 cells of the ETaR siRNA group were lower than those of the Hypoxia siRNA group, while such results were much higher in the L-NAME group. Western Blot results showed lower expression of ETaR in the ETaR siRNA group compared with the hypoxia and negative siRNA groups, as well as significantly higher ETaR expression in the L-NAME group compared with the ETaR siRNA group. PI3K and p-AKT expression levels were mildly elevated after mild oxygen deprivation, and ETaR siRNA was able to enhance such elevation induced by hypoxia. In the L-NAME group, PI3K

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and p-AKT expression was much higher than the ETaR siRNA group. PKG and sGC expression levels significantly descended after mild oxygen deprivation. While such levels were higher in the ETaR siRNA group, compared with the hypoxia and negative siRNA groups, the L-NAME group had lower levels of PKG and sGC compared with the ETaR siRNA group. **Conclusion:** ETaR siRNA is capable of down-regulating the expression of inflammatory and transcription factors among endothelial cells treated with hypoxia. Down-regulation of ET-1 is triggered by altered nucleus transcription factor activity through the sGC/PKG signal pathway, and results in enhanced eNOS activity through the PI3K/Akt signal pathway. We suspect this to be the mechanism of the protective effect of ETaR siRNA.

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Introduction

Ischemia reperfusion is an inevitable process during kidney transplantation, the ischemia reperfusion injury (IRI) caused by such a process is an important reason for renal graft impairment, the mechanisms of which remains unclear [1]. There is also no positive cure for kidney graft IRI. Research on kidney graft IRI development, prevention and treatment is thus of essential clinical importance. The pathophysiological change observed in renal IRI includes mainly cell death, altered hemodynamics, oxidative stress, inflammation etc [2]. In early ischemia reperfusion, excessive contraction of renal blood vessels and injury of vascular endothelial cells [3] result in vascular endothelial over-expression of adhesion molecules, as well as increase in cytokines including TNF- α , IL-6 etc. This results in renal tubular inflammation infiltration and activation of compliments [4]. Inflammation cells further enhance kidney graft damage with reactive oxygen, protease as well as inflammation factors produced through elevated mechanical resistance of blood capillaries. Excessive contraction of renal blood vessels and vascular endothelial damage are recognized pathophysiological characteristics of kidney graft IRI. Thus, the search for a safe and effective method to access early prevention of excessive contraction of renal blood vessels and vascular endothelial damage is of great importance in the pursuit of long-term kidney graft survival.

Endothelin (ET) remains the strongest endogenous vasoconstrictor substance found. It regulates renal blood flow, glomerular filtration rate, sodium and water metabolism and acid-base balance [5]. There are three known subtypes of ET: ET-1, ET-2 and ET-3. ET-1 is mainly produced by endothelial cells, and its expression is significantly increased after renal ischemia reperfusion [6]. ET implies its biological effects by combining with endothelin receptors (ETR), including endothelin A receptors (ETaR) and endothelin B receptors (ETbR). ETaRs are found mostly on the surface of vascular smooth muscles, and ETbRs on the surface of endothelial cells [7]. When ET-1 conjuncts with the ETaR on vascular smooth muscle cells, blood vessels contract and cause spasm, enhancing renal ischemia. However, when ET-1 conjuncts with the ETbR on the surface of endothelial cells, nitric oxide (NO) and prostaglandins are produced and blood vessels are dilated [7]. Studies have indicated that nonselective endothelin receptor antagonist effectively normalized renal function in diabetic nephropathy [8]. However, blocking both ET_AR and ET_BR caused long-term renal dysfunction in IRI model [9]. As a result, inhibition of ETaR expression is considered a novel method in the treatment of renal ischemia reperfusion injury.

Exogenous small interfering RNA (siRNA) can effectively and specifically inhibit target gene expression of mammal cells. In earlier research, we found that ETaR-specific siRNA can suppress renal ETaR expression, mitigate renal structural injury, inhibit local inflammation factor expression, and improve renal function in rat renal ischemia and reperfusion injury models [10]. Further studies show that decrease of ETaR expression is capable of repressing local ET-1 expression through feedback inhibition, which may be an important way of protection against renal ischemia and reperfusion injury [10]. Previous studies show that ETaR siRNA can specifically inhibit local ETaR expression in kidneys, resulting in a significant decrease in ET-1 mRNA expression as well as elevated local NO levels, providing protection against renal ischemia and reperfusion injury [10]. Thus, we propose the hypothesis as

follows:

(1) ETaR siRNA decreases the ETaR expression on the surface of smooth muscles, increasing the chance of conjunction of ET-1 and the ETbR on the surface of endothelial cells, thus increasing endothelial production of NO through the PI3K/Akt pathway.

(2) In an endothelial cell, ET-1 expression may be suppressed by NO through activity regulation of nuclear transcription factors HIF-1, AP-1, NF- κ B and Smad using the sGC/cGMP/PKG pathway.

(3) NO may also decrease ET-1 expression by relaxing vascular smooth muscle, improving the status of hypoxic tissue and inhibiting nuclear transcription factor HIF-1.

In this study we aim to clarify this mechanism through *in vitro* and *in vivo* experiments.

Materials and Methods

Cell and reagents: The rat endothelial and smooth muscle cells used were purchased from ATCC. The ETaR siRNA and Negative ETaR siRNA used in this study were synthesized by Shanghai Gene Pharma Co., Ltd. The Transwell six-well plates were purchased from CORCOR co. Dulbecco Modified Eagle Medium (DMEM) High Glucose and fetal bovine serum (FBS) were purchased from Gibco Technologies (Logan, UT, USA). Trizol reagent, superscript II reverse transcriptase and random primer oligonucleotides were purchased from Invitrogen (Carlsbad, CA, USA). The Absolute QPCR SYBR Green premix was purchased from Takara (Otsu, Shiga, Japan). The sequences of primers used in this study are presented in Table 1. The antibodies against PI3K, p-AKT, ETaR, PKG and GAPDH were purchased from Cell Signaling (Beverly, MA, USA), and the antibodies against β -actin and cGMP were purchased from Abcam (Abcam, MA, USA).

Cell culture and transfection: Rat smooth muscle cells and vascular endothelial cells were cultured under 37°C, 5%CO₂ and humid conditions. Medium was changed and cells were passaged regularly. Transfection of synthesized ETaR siRNA into rat vascular endothelial cells was performed using a Lipofectamine 3000 Transfection Kit, according to the instructions. Medium was changed routinely 4 to 6 hours after transfection.

Establishment of slight hypoxic model: The rat smooth muscle cells and vascular endothelial cells cultured were seeded respectively in the upper and lower layers of the Transwell six-well plates for co-culture. ETaR siRNA was transfected into rat vascular smooth muscle cells (A-10 cells were chosen in this study) before hypoxic treatment, which was carried out in an 37°C, 1%O₂ incubator after a routine change of medium. 2.6ml of endothelial cellular culture medium was added to the lower layer of each well in the six-well plates, and 1.6ml of high-glucose cellular culture medium was added to the upper layer. The six-well plates were collected and stored for 24hrs under a normal 37°C, 5%CO₂ and humid culture condition at 0.5h, 1h, 2hrs, 4hrs, 8hrs, 16hrs, 24hrs and 48hrs of hypoxia. Both types of cells as well as endothelial cell supernatant samples were collected for further examination. 5 groups were established: (1) normal group (Nor group); (2) hypoxic group (Hypoxia group); (3) ETaR Negative siRNA hypoxic group (Neg siRNA group); (4) ETaR siRNA group (siRNA group); (5) ETaR siRNA+ L-NAME group (L-NAME group).

Table 1. The sequence of gene-specific primers.

Gene	The Sequence of Gene-Specific Primer	Gene	The Sequence of Gene-Specific Primer
ETaR	F -ACATGCTGGCTGGTTCTGGAG- R -ATGGGCAGGAGTGTAGCCAGTC-	TNF- α	F -CCTTATCTACTCCCAGGTTCTC R -AGGGGCCATCCACAGTCTTTC
NF- κ B	F -CACTTAGCCATCATCCACCTTC- R -AGTCTCCACCATCTTCC	TGF- β	F -CTTGCCCTCTACAACCAACA R -CTTGCGACCCACGTAGTAGA
HIF-1	F -CCCTACTATGTCGCTTTCTTGG R -GGTTTCTGCTGCCTTGTATGG	ANG-II	F -CTGAGGCAAACTGCGGCAAG R -GTAGATGGCGAACAGGAACG
Smad-2	F -CACTCACTGTAGACGGCTTTAC R -GCACATCACTTAGGCACTCG	ET-1	F -ACCTGGACATCATCTGGGTCAAC R -TTTGGTGAGCACACTGGCATC
Smad-3	F -AGGAGAAGTGGTGCAGAAAG R -CACAAAGCGGCAGTAGATGAC	MCP-1	F -CTATGCAGGTCTCTGTACGCTTC R -CAGCCGACTCATTGGGATCA
IFN- γ	F -ATGGATGCTATGGAAGGAAAGAG R -CACTTATGTTGTTGCTGATGGC	IL-6	F -CCACTTCACAAGTCGGAGGCTTA R -GTGCATCATCGCTGTTTACATAATC

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from rat vascular endothelial and smooth muscle cells with Trizol reagent. 1 µg of total RNA was reverse transcribed into cDNA using a Invitrogen cDNA Synthesis Kit. The primers are shown in Table 1. qRT-PCR was performed using the SYBR Premix. After a hot start (30 seconds at 95°C), amplification was performed for 40 cycles (5 seconds at 95°C, 30 seconds at 55°C, 60 seconds at 72°C). Expression levels were normalized relative to those of β-actin in the same samples using the 2-ΔΔCt method.

ELISA

Endothelial cell supernatant was retrieved and centrifuged at 2000rpm for 20 min, after which supernatant was carefully collected. Supernatant or standard samples were added to ELISA plates, mixed and left at 37°C for 40min according to the introductions of Westang Biotech Elisa kit. Primary antibodies, enzyme labeled antibody and substrate working solution were added respectively after washing off. Absorbance was measured at 450nm using a microplate reader after adding the stop solution. Using standard concentrate gradient as abscissa and OD value as ordinate, a graph was drawn to show the corresponding amount of sample.

Western Blot analysis

After washing the co-culture cells with PBS twice, vascular endothelial and smooth muscle cell protein was collected following the instructions of the Beyotime protein retrieving kit. Western Blot analysis was carried out as previously described [11]. Expression of ETaR, PI3K, p-AKT, sGC, PKG were quantitated by normalizing to reference proteins (β-actin or GAPDH) using Image-Pro plus 6.0 software.

Animal model:

Lab animals: Male SPF grade SD rats, their body weight varying from 200-220g, were obtained from Shanghai Slac Lab Animal Co. Ltd. and bred in an SPF grade animal room. All animal procedures were performed according to the guidelines of the Care and Use of the Laboratory Animal Ethical Commission of Fudan University. The rats were randomly divided into 4 groups of 6 individuals: Sham, IR, Negative siRNA and R+ETaR siRNA group. The renal pedicles of the laboratory animals were clipped and drugs were given through deep venous hypertensive perfusion. The kidney IR model was established as previously described [12], while

Fig. 1. A and B: Compared to the hypoxic group, the ETaR mRNA of the smooth muscle cells and the ET-1 mRNA of the endothelial cells within the ETaR siRNA group were statistically lower in varying degrees. C: In comparison with the hypoxic group, the amount of ET-1 in the co-culture supernatant of the ETaR siRNA group was statistically lower at 2hrs, 4hrs, 8hrs, 16hrs, and 24hrs. D and E: The amount of supernatant NO and eNOS of the ETaR siRNA co-culture group was statistically higher at many time points, compared to the hypoxia group.

Fig. 2. After 4hrs of hypoxia, the mRNA of the co-cultured endothelial cells were extracted. After retro-transcription, PCR results showed significant increase in Smad2, Smad3, HIF-1, NF-kb, TNF- α , IFN- γ , IL-6, MIP-2, MCP-1, ANG II and ET-1 expression within hypoxic cells, compared to the control group. ETaR siRNA significantly lowered the inflammatory and transfection factors mention above, while in contrast, negative siRNA application did not result in such an effect.

blood samples and kidneys were collected 2 days after model establishment. Renal function was compared between groups using serum creatinine and urea nitrogen levels achieved from automatic biochemical analyzers. Kidney tissue was fixed in formaldehyde and embedded in paraffin. Damage was checked using common HE staining.

Results

ETaR siRNA inhibited the expression of ETaR in smooth muscle cells, and was able to decrease the ET-1 expression of endothelial cells through eNOS-related signal pathway. In order to detect the effect of chemically synthesized ETaR siRNA, we established a hypoxic rat vascular endothelial-smooth muscle cell co-culture model. Evaluation of ET-1 mRNA level in smooth muscle and endothelial cells at 0.5hrs, 1h, 2hrs, 4hrs, 8hrs, 16hrs, 24hrs and 48hrs revealed that as the time of hypoxia lengthened, the expression of ETaR and ET-1 mRNA rose. By comparing with the hypoxic group, one could conclude that the use of ETaR siRNA inhibits the ET-1 mRNA levels of smooth muscle and endothelial cells. Such effect was most significant at 4hrs of hypoxia. Supernatant ET-1, NO and eNOS was analyzed using ELISA.

Fig. 3. Protein of co-cultured endothelial cells were extracted after 4hrs of hypoxia. Western Blot analysis showed that PI3K and p-AKT levels were slightly elevated within the hypoxia group, this effect enhanced with ETaR siRNA application. Compared with the control group, the sGC and PKG levels of the hypoxia group were significantly lower, while those of the ETaR siRNA group were higher. Introduction of Negative siRNA didn't result in change in the hypoxia group.

Results showed that ETaR siRNA application decreased the amount of ET-1 and increased the amount of NO and eNOS in the supernatant at all times of hypoxia. The difference was of statistical significance at most time points.

ETaR siRNA inhibits the inflammatory and transfection factors of hypoxic endothelial cells

To investigate the molecular mechanism of ET-1 feedback inhibition, we placed the co-culture system under hypoxic system for 4 hrs. Inflammatory and transfection factor expression levels within endothelial cells were detected, and a Negative siRNA group was added. After 4 hrs of hypoxia, a significant rise in Smad2, Smad3, HIF-1, NF-kb, TNF- α , IFN- γ , IL-6, MIP-2, MCP-1, ANG II and ET-1 expression was observed. This phenomenon can be changed by ETaR siRNA application, while Negative siRNA application does not possess such an ability.

ETaR siRNA activates PI3K/AKT and sGC/cGMP/PKG signaling pathway

The PI3K/AKT and sGC/cGMP/PKG signaling pathway was analyzed using Western Blot. Results showed that after 4hrs of hypoxia, the PI3K and PKG levels of endothelial cells

Fig. 4. Compared to the hypoxia group, the mad2, Smad3, HIF-1, NF-kb, TNF- α , IFN- γ , IL-6, MIP-2, MCP-1, ANG II and ET-1 expression of the EtaR siRNA group was significantly lower. The mRNA levels of such factors within the L-NAME group were statistically higher, equivalent to the hypoxia group.

were slightly elevated. This elevation was magnified by the use of ETaR siRNA. sGC and PKG levels were significantly lowered after hypoxia, and the use of ETaR siRNA caused a rise in the indicators mentioned. Negative siRNA application didn't cause difference in the hypoxia group.

eNOS inhibitor L-NAME reverses the decrease of inflammation and transfection factors caused by ETaR siRNA

We used an eNOS inhibitor L-NAME for further experiment, as it acts as a connecting particle within the signaling pathway mentioned above. Results showed that ETR siRNA application lead to a significant decrease in the inflammation and transfection factors mentioned above caused by hypoxia, while the mRNA levels of such factors within the L-NAME group were much higher than that of the ETaR siRNA group and parallel with the hypoxia group.

eNOS inhibitor L-NAME blocks ETaR siRNA activated PI3K/AKT and sGC/cGMP/PKG signaling pathway

We used Western Blot analysis to further confirm the feedback mechanism by adding eNOS inhibitor L-NAME to the system. It was discovered that the PI3K and P-AKT levels of

Fig. 5. At 4hrs of hypoxia, co-cultured endothelial cellular protein was extracted. Western Blot results showed that the PI3K and p-AKT levels of the hypoxia group were lower than that of the ETaR siRNA group and higher than that of the L-NAME group. sGC and PKG levels of the hypoxia group were lower than that of the ETaR siRNA group and higher than that of the L-NAME group.

the endothelial cells within the ETaR siRNA group were higher than those of the hypoxia group, while additional L-NAME caused such levels to decrease to various extents. sGC and PKG levels were lowered after hypoxia. This effect could be reversed by the introduction of ETaR siRNA. Additional ETaR siRNA and L-NAME also caused such levels to decrease.

ETaR siRNA decreases rat kidney damage among rats.

We examined the IR kidney model. 48h after surgery, Scr and Urea nitrogen levels were evaluated and HE staining was performed to determine the amount of injury. We observed an increase in Scr and Urea nitrogen levels. Among all groups, the renal function of the ETaR siRNA group was clinically significantly decreased.

The use of Negative siRNA did not result in IR-induced deterioration of renal function. Rat kidney samples showed significant damage at 48h post IR surgery, including tubular dilation, cast formation, loss of brush-like edge and epithelial cell vacuolar degeneration. Renal injury was lightest in the ETaR siRNA, while Negative siRNA was not effectively protective.

Fig. 6. 48h after IR treatment, the Scr and Urea nitrogen levels of the ETaR siRNA group was statistically lower compared to the other groups, while that Negative siRNA group remained average. Pathological damage was obvious 48h after IR, showing tubular dilation, cast formation, loss of brush-like edge and epithelial cell vacuolar degeneration etc. Renal injury was lightest in the ETaR siRNA, while Negative siRNA did not show to be protective.

Discussion

A kidney transplant is the best way to treat end stage renal disease. Ischemia reperfusion injury affects the recovery of graft function, influencing long term graft function and survival [13]. Disorder of the ET system is a characteristic pathophysiological change in renal transplant graft ischemia reperfusion injury, and regulation of the ET system can lead to sufficiently controlled kidney graft injury [14]. ET-1 expression is affected by numerous factors: TNF- α , INF- γ , TGF- β , ANG II and hypoxia increase ET-1 expression; NO and prostaglandin down-regulate ET-1 expression [15, 16]. The regulative media-related nuclear transcription factors mentioned above play a key role in ET-1 gene transcription.

In this study, a hypoxic rat vascular smooth muscle-endothelial cell co-culture model was established. The effect of ETaR siRNA induced decreased ETaR expression on ET-1 was observed, as was inflammation and transfecting factors within the co-culture system.

TNF- α can cause damage of vascular endothelial cells, result in vascular injury and malfunction, inducing thrombosis [17, 18]. It can also enhance neutrophil phagocytosis through antibody-dependent cell-mediated cytotoxicity (ADCC), promote neutrophil attachment to vascular endothelial cells, and result in the production of oxygen free radicals and cellular death. This stimulates local inflammation, aggravating IR injury [19]. Research have shown that the Effects of TNF- α can be attenuated in vascular endothelial cells by causing ectodomain shedding of TNF receptor 1 [20]. Smads mediates the transfer of TGF- β signal from the surface of cells to the nucleus. Activated Smads enters the nucleus, activating the transfection of down-stream target genes, elevating local inflammation [21]. Interleukin-6 (IL-6) can stimulate the growth and differentiation of immune response related cells, enhancing their function [22]. Hypoxia inducible factor-1 (HIF-1) is a nuclear protein with transfecting activity. Activated HIF-1 participates in cellular survival and apoptosis, as well as biological effects such as drug resistance. Overproduction of HIF-1 increases local inflammation and aggravates IR injury [23]. Interferon- γ (IFN- γ) is a secretive factor with the ability to induce macrophage iNOS production, the overproduction of which is important in pathological processes including inflammation and acute rejection [24]. MCP-1 is a type of CC chemokine. It's main function is the chemotaxis and activation of macrophages [25]. NF- κ b responds to harmful stimuli at once, regulating molecules that participate in early immunological reaction and all stages of inflammation [26]. The quantity of the inflammation and transfection factors mentioned above were detected, and we found that the Smad2, Smad3, HIF-1, TNF- α , IFN- γ , IL-6, MCP-1, NF- κ b and ANG II mRNA levels of endothelial cells in the co-culture model were lower than those of the IR and Hypoxia groups, while the application of eNOS inhibitor L-NAME seemed to reverse such effects. This indicates that the effect of ETaR siRNA is closely related to eNOS.

NO, the first *in vivo* gas-form intracellular information molecule to be found, is a non-classic neurotransmitter. NOS catalyzes the transformation from L-arginine to NO. NOS can be divided into neuro NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), among which nNOS is located mainly in the nervous system. The kidney contains mostly eNOS, located in tubular epithelial and vascular endothelial cells [27-29]. Research show that iNOS originates mainly from macrophages in tissue. It participates in tissue inflammation, high expression of which elevates renal injury [30]. eNOS participates in the regulation of renal blood flow, prevents spasm of renal blood vessels and decreases infiltration of inflammatory cells [31].

ELISA results revealed significantly increased NO and decreased eNOS expression in hypoxic endothelial cells. This indicates a connection between the increased NO and elevated iNOS. NO levels were further elevated after ETaR siRNA treatment, as was eNOS in comparison with the positive control group. One can presume that siRNA treatment can increase eNOS levels, cause more NO production, relax blood vessels, and possibly provide a renal protecting effect by regulating renal blood flow. The fact that the use of eNOS inhibitor L-NAME reverses the protective effect brought by siRNA further indicates that such protective effect is a result of an increased amount of NO realized through eNOS regulation.

Fig. 7. ETaR siRNA decreases the ETaR expression on the surface of smooth muscles, increasing the chance of conjunction of ET-1 and the ETbR on the surface of endothelial cells, thus increasing endothelial production of NO through the PI3K/Akt pathway. In endothelial cell, ET-1 expression was suppressed by NO through activation of nuclear transcription factors, such as HIF-1, AP-1, NF-κB and Smad via the sGC/ cGMP/ PKG pathway.

The PI3K/ Akt signaling pathway regulates cellular proliferation, inflammatory response and chemotaxis. It plays an important part in cell life and apoptosis. Research shows that renal ischemic preconditioning and ischemia reperfusion itself stimulates the PI3K/Akt pathway [32, 33]. Our results confirmed this phenomenon, as a slight rise in PI3K and p-AKT was observed in hypoxic vascular endothelial cells. ETaR siRNA treatment caused PI3K and p-AKT to significantly rise, further activating the eNOS on vascular endothelial cells, dilated blood vessels (Fig. 7).

PI3K expression levels remained the same, but p-AKT levels significantly decreased with L-NAME application, possibly related to the fact that L-NAME acts on the downstream rather than upstream of the eNOS pathway. As the downstream of the NO signaling pathway, sGC/cGMP/PKG regulates the activity of various transfecting factors, including HIF-1, AP-1, NF-κB, Smad etc [34]. We discovered that sGC and PKG protein levels within endothelial cells decreased with hypoxia, increased with additional ETaR siRNA treatment, and decreased again with the use of L-NAME. This suggests that ETaR siRNA causes additional NO by activating eNOS through the PI3K/AKT pathway and further acting on the sGC/cGMP/PKG signaling pathway to regulate downstream transfecting factors (Fig. 7).

To further test the effect of ETaR siRNA *in vivo*, a rat renal ischemia and reperfusion model was established. The renal pedicles of the laboratory animals were clipped and drugs were given through deep venous hypertensive perfusion. Blood and tissue samples retrieved 48h after IR treatment revealed increased Scr and Urea nitrogen levels and significant pathological damage. Renal injury was lightest in the ETaR siRNA group, indicating its possible protective effect *in vivo*, while Negative siRNA was absent of this ability.

Also, ETaR siRNA was found capable of inhibiting hypoxia-induced ET-1 and ETaR elevation. As this effect was reversed by introduction of L-NAME, one can speculate that ETaR siRNA inhibits downstream expression of ET-1 by decreasing ETaR expression and regulating a series of pathways, ultimately providing protection against hypoxic injury.

Conclusion

In the current study, we found that ETaR siRNA is capable of down-regulating the expression of inflammatory and transcription factors among endothelial cells treated with

hypoxia. The protective effect of ETaR siRNA may be accessed through the sGC/PKG and PI3K/Akt signal pathway. In the meantime, ETaR siRNA has potential in inhibiting tissue injury as well as ameliorating renal function impairment.

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