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ET-1 deletion from endothelial cells protects the kidney during the extension phase of ischemia/reperfusion injury

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ABSTRACT

Background: The prognosis of patients after acute kidney injury (AKI) is poor and treatment is limited. AKI is mainly caused by renal ischemia/reperfusion injury (IRI). During the extension phase of IRI, endothelial damage may participate in ischemia and inflammation. Endothelin-1 (ET-1) which is mostly secreted by endothelial cells is an important actor of IRI, particularly through its strong vasoconstrictive properties. We aimed to analyze the specific role of ET-1 from the endothelial cells in AKI.
Methods: We used mice lacking ET-1 in the vascular endothelial cells (VEETKO). We induced IRI in VEETKO mice and wild type controls by clamping both kidneys for 30 min. Sham operated mice were used as controls. Mice were sacrificed one day after IRI in order to investigate the extension phase of IRI. Kidney function was assessed based on serum creatinine concentration. Levels of expression of ET-1, its receptor ET_A, protein kinase C, eNOS, E-Cadherin and inflammation markers were evaluated by real time PCR or western blot. Tubular injury was scored on periodic acid Schiff stained kidney preparations. Lumen and wall area of small intrarenal arteries were measured on kidney slices stained for alpha smooth muscle cell actin. Oxidative stress, macrophage infiltration and cell proliferation was evaluated on slices stained for 8-hydroxy-2′-deoxyguanosine, F4/80 and PCNA, respectively.
Results: IRI induced kidney failure and increased ET-1 and ET_A receptor expression. This was accompanied by tubular injury, wall thickening and reduction of lumen area/wall area ratio of small renal arteries, increased oxidative stress and inflammation. These parameters were attenuated in VEETKO mice.
Conclusion: Our results suggest that suppression of ET-1 from the endothelial cells attenuates IRI kidney injury. Blocking ET-1 effects may represent a therapeutic strategy in the management of AKI.

1. Introduction

Acute kidney injury (AKI) is a common clinical problem with a high mortality rate, predominantly in intensive care units [1]. The prognosis of patients after AKI is poor and treatment is limited [2]. Renal ischemia/reperfusion injury (IRI) represents the most frequent cause of AKI and leads to chronic progression in up to 70% of the cases [3].

Reduction of renal perfusion due to an imbalance between renal vasoconstriction and vasodilatation mediators is believed to play a role in IRI and its chronic complications [4]. Together with the hemodynamics changes, inflammation and tubular epithelial injury are major components of the pathophysiology of AKI [3]. The role of the endothelial cells from the microvasculature in AKI is important, particularly in their ability to regulate inflammatory cell infiltration [5] and tubular function [6].

Endothelin-1 (ET-1) that is mostly secreted by endothelial cells is a potent vasoconstrictor [7]. ET-1 infusion in perfused kidney induces reduction of renal blood flow and glomerular filtration rate through its vasoconstriction effect [8].

ET-1 is up-regulated in the renal ischemic period [6]. Blocking the endothelin system using selective and non-selective receptor blockers as well as drugs reducing ET-1 production is efficient in reducing the damage to the kidney caused by IRI [9–12]. Nevertheless, systemic blockade of ET-1 receptors induces undesirable side effects which limit their clinical use [13].

In the present study, we have addressed the question whether blocking specifically ET-1 from the vascular endothelial cells (EC) is sufficient to prevent IRI-induced renal damage. We have focused on the extension phase of kidney IRI, the phase in which the damage of the vascular endothelial cells may be responsible for inflammation and tubular epithelial injury [14–16].

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2. Material and methods

2.1. Animal experiment and kidney IRI model

We used mice with vascular EC-specific ET-1 knock-out (VEETKO) and their wild type (WT) littermates as described previously [17]. The mice were housed in 12-h light and dark cycle with free access to water and chow. Four-month old male VEETKO mice and WT littermates (each $n = 8$) were subjected to kidney IRI. Experiments were conducted by following the established guidelines for animal care of the Kobe University.

The surgical preparation and operation methods were previously described [18]. Briefly, mice were anesthetized using inhalational isoflurane (Merck). Abdomen was opened and both renal pedicles were clamped using non-traumatic microaneurysm clamps (Roboz Surgical Instrument, RS5426) for 30 min. Mice were placed on a 37 °C heating pad during ischemic period. After removal of the clamp, the abdomen was clospage 5ed. Sham operated (SO) mice ($n = 5$) underwent similar procedures except for renal pedicles clamping. For sacrifice, mice were anesthetized with pentobarbital (60 mg/kg ip), and then abdomen and thorax were opened. Organs were perfused with 0.9% NaCl. Renal tissues were harvested, snap frozen for RNA and protein extraction, embedded in OCT compound (SAKURA, 4583, Japan) and fixed in 4% paraformaldehyde for paraffin embedding. For RNA and protein extraction, kidneys were divided into cortex and medulla. We sacrificed the mice on the day following the operation to examine the extension phase of kidney IRI.

2.2. Kidney function assessment

Blood was collected from the orbital sinus before sacrifice. 0.8–1 mL of blood was collected in an Eppendorf tube, incubated one hour at room temperature, and finally centrifuged at 3000 rpm for 10 min. Serum was collected and kept at −80 °C. Serum creatinine level was measured using the ELISA kit Nescoat VLII CRE (Alfresa Pharma Corp., Japan).

2.3. Histological analysis

Four-mm paraffin sections were de-paraffinized, and stained with periodic acid Schiff's reagent (PAS) to evaluate tubular injury. Immunohistochemical (IHC) staining was done for these following antibodies: F 4/80 (1:100; AbDSerotec, MCA497), PCNA (1:200; DAKO, N1529) and 8-Hydroxy-2′-deoxyguanosine (8-OHdG) (1:50; JaICA, Japan, MOG-100P). Briefly, paraffin sections were de-paraffinized, heated in citrate buffer (10 nM Sodium Citrate, 0.05% Tween20 pH 6), then incubated in 3% H_2O_2. Antibodies were incubated overnight after blocking with 3% bovine serum albumin in phosphate buffered solution (PBS), TritonX 0.05%. These following secondary antibodies were used and incubated for one hour: Anti mouse Dako Envision labeled Polymer-HRP (DAKO, K4000) for proliferating cell nuclear antigen (PCNA) and 8-OHdG, and Histofine anti-Rat (Nichirei, 414311F) for F4/80.

For immunofluorescence (IF) staining, a monoclonal antibody anti alpha-smooth muscle actin (α-SMA) FITC conjugate was used (Sigma, F3777, 1:250) and a polyclonal anti-ET_AR antibody (IBL, 16202, 1:10) De-paraffinized kidney sections were heated in citrate buffer, and incubated in proteinase K (1:20, Dako, 2010–02) for 20 min at 37 °C, then washed in PBS, Triton 0.05%. 1% BSA in PBST was used as blocking and antibody dilution buffer. An alexa Fluor 568 conjugated anti-rabbit IgG antibody (1:250, Invitrogen, A10042) was used as secondary antibody for ET_AR.

2.4. Tubular injury score

Tubular injury was scored based on PAS staining. Scoring was done by grading tubular injury and dilatation, intra-luminal cash and brush border loss in fifteen randomly chosen, non-overlapping fields (200× magnification). The lesions were graded on a scale from 0 to 4: 0: normal; 1: the injury involve less than 25% of the cortex; 2: the injury involve 25 to 50% of the cortex; 3: the injury involve 50 to 75% of the cortex; and 4: the extensive injury involving more than 75% of the cortex [19].

2.5. Wall thickness of intra-renal arteries

Wall thickness was quantified based on αSMA IF in intra-renal arteries (diameter = 10–50 μm). Fifteen to twenty arteries were randomly chosen, photographed using a Keyence Immunofluorescence microscope. Using the Image J software, we measured the vessel and lumen area; wall area was measured by subtracting lumen area from vessel area and the ratio between wall and lumen area was calculated. We then measured wall and lumen perimeters; wall thickness was measured as the wall area normalized to the mean of vessel and lumen perimeters.

2.6. Real-time PCR

Total RNA was isolated from whole kidney and cortex tissue using Trizol (Invitrogen, 1559–018). To obtain cDNA, 1 μg of RNA was used for reverse transcription based on the manufacturer's instructions (ReverTra, TOYOBO, TRT-101). Quantitative real-time PCR was performed using a Thunder bird SYBR® qRCR Mix (TOYOBO, QPS-201) on an ABI 7500 RT thermocycler. The following conditions were used for amplification: 95 °C for 10 s, 40 cycles at 95 °Cfor 5 s, 60 °C for 34 s. Quantification of gene expression was performed using the delta-delta C_T method considering HPRT-1 as a house keeping gene. The following primers were used: ET-1 (forward, 5′-TGCTGTTCGTGACTTTCC-3′; reverse, 3′-TGTTGACCCAGATGATGTC-5′), ET_AR (forward, 5′-GCTGGTTCCCTCTTCACTTAAGC-3′; reverse, 3′-TCATGGTTGCCAGGTTAATGC-5′), MCP-1 (forward, 5′-GGCATCACAGTCCGAGTCACA-3′; reverse, 3′-CTACAGACAACCACCTCAAGCACTTC-5′), TLR2 (forward, 5′-AAGAAGCTGGCATTCCGAGGC-3′; reverse, 3′-CGTCTGACTCCGAGGGGTTGA-5′), TLR4 (forward, 5′-GGGCCTAAACCCAGTCTGTTTG-3′; reverse, 3′-GCCCGGTAAGGTCCATGCTA-5′), and ICAM-1 (forward, 5′-CAATTCACACTGAATGCCAGCTC-3′; reverse, 3′-CAAGCAGTCCGTCTCGTCCA-5′), HPRT-1 (forward, 5′-TTGTTGTTGGATATGCCCTTGACTA-3′; reverse, 3′-AGGCAGATGGCCACAGGACTA-5′) was used as reference.

2.7. Western blotting

40 μg proteins extracted from kidney tissues were separated on 8% SDS–PAGE, transferred to a polyvinylidene fluoride membrane, and probed with E-Cadherin polyclonal antibody (ABCAM, ab15148), eNOS (BD Pharmingen, 610296), ET_AR (Santa Cruz, Sc-33536), PKC (Santa Cruz, SC-1681) and GAPDH antibody (Sigma, G9545). 5% skim milk in TBST was used for blocking before first antibodies incubation. For E-cadherin, eNOS, ET_AR, and GAPDH, a HRP linked anti-rabbit IgG antibody (1:3000, Cell Signaling, #7074) and, for PKC, a HRP linked anti-mouse IgG (1:4000; Jackson Immunoresearch, 315–035-003) were used as secondary antibodies. Proteins were visualized using a Luminata Forte Western HRP Substrate (Millipore, WBLUF0100). Blots were photographed with a transilluminator LAS 3000 mini (Fuji Film) and quantified by densitometry using Image Reader LAS 3000 mini.

2.8. Reactive oxygen species (ROS) detection by dihydroethidium (DHE) staining

Fresh kidneys were embedded in OCT compound (SAKURA, 4583, Japan), and then cut into 4-μm thick slices using cryostat (Leica, CM3050). The cryosections were washed in PBS and incubated in 1 mM of DHE (Molecular Probe, D23103) for 30 min. Red fluorescence was visualized at 567 nm with a Keyence microscope and pictures were taken with a digital camera. Fifteen randomly non-overlapping fields (200× magnification) in each sample were analyzed. DHE intensity was measured using Image J software.

2.9. Statistics

Results were expressed as mean ± SD. Differences between groups were analyzed by ANOVA and *t*-test using the STATVIEW software. Difference between groups were considered statistically significant at a P value < 0.05.

3. Results

3.1. ET-1 deletion from EC attenuated renal failure after IRI

Serum creatinine level increased dramatically one day after IRI indicating renal failure. VEETKO mice presented a significantly lower serum creatinine level than WT mice (Fig. 1A).

3.2. ET-1 deletion from EC attenuated cortex tubular injury after IRI

There were no histopathological differences between SO WT and VEETKO mice. IRI induced tubular injury in both genotypes but VEETKO mice had a significantly lower tubular injury score than WT mice (Fig. 1B and C). In WT mice, the injury spread to the cortex more extensively compared to VEETKO mice (data not shown). PCNA immunostaining showed an increased positive signal in epithelial cells one day after IRI indicating cell proliferation (Fig. 1D and E). E-Cadherin expression decreased after IRI (Fig. 1F). ET-1 deletion from EC increased the number of PCNA positive epithelial cells and increased E-Cadherin protein abundance in renal tissue (Fig. 1D–F).

3.3. ET-1 and ET_AR expression increased after IRI

IRI induced ET-1 expression in whole kidney and cortex. Endothelial cell-derived ET-1 deletion significantly reduced mRNA ET-1 level (Fig. 2A and B). Similarly, IRI-induced increase of ET_AR mRNA and ET_AR protein level was stronger in WT than in VEETKO mice (Fig. 2C and D).

3.4. ET-1 deletion from EC attenuated IRI–induced intrarenal artery wall thickening

IRI induced an increase of the wall thickness of small renal arteries in WT but not in VEETKO mice. Lumen/wall area ratio decreased in both genotypes after IRI. In VEETKO mice it remained

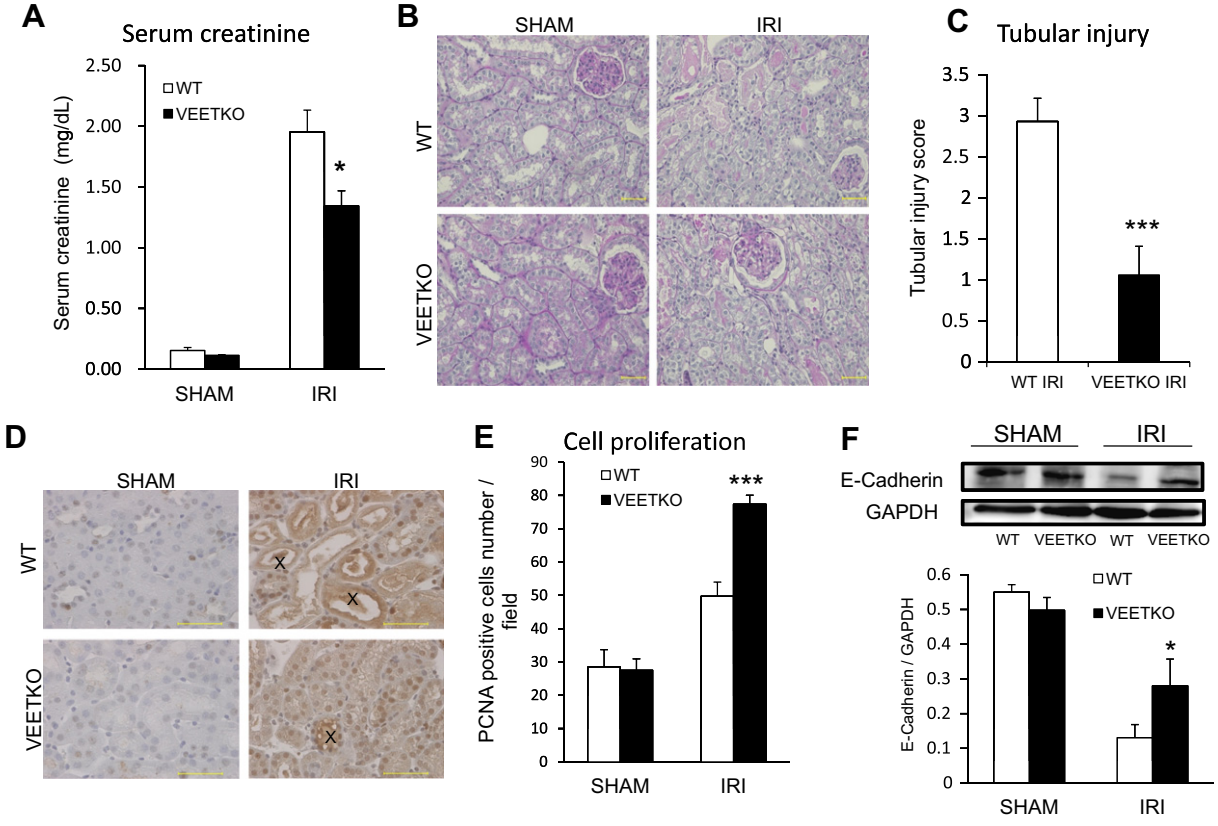

Fig. 1. Effect of ET-1 deletion from EC on kidney function and histology and cell proliferation one day after IRI. (A) Kidney function was assessed based on serum creatinine concentration. (B and C) Tubular injury was scored on PAS stained renal sections. (B) Representative images (200× magnification, 12 fields each sample, $n = 6$). (C) Tubular injury score. (D-E) Cell proliferation was assessed based on renal sections stained for PCNA. (D) Representative images of PCNA staining (800× magnification, 15 field each sample, $n = 5$). Epithelial cells showed positive signal. Staining in the intra-luminal cast (X) was due to unspecific binding of the secondary antibody. (E) Quantification of PCNA positive cell number. (F) Western blot and densitometric analysis of the epithelial cell marker E-Cadherin ($n = 4$). Results were expressed as means ± SD. $^{*}P < 0.05$, $^{***}P < 0.001$ versus WT IRI. Bar = 50 μm.

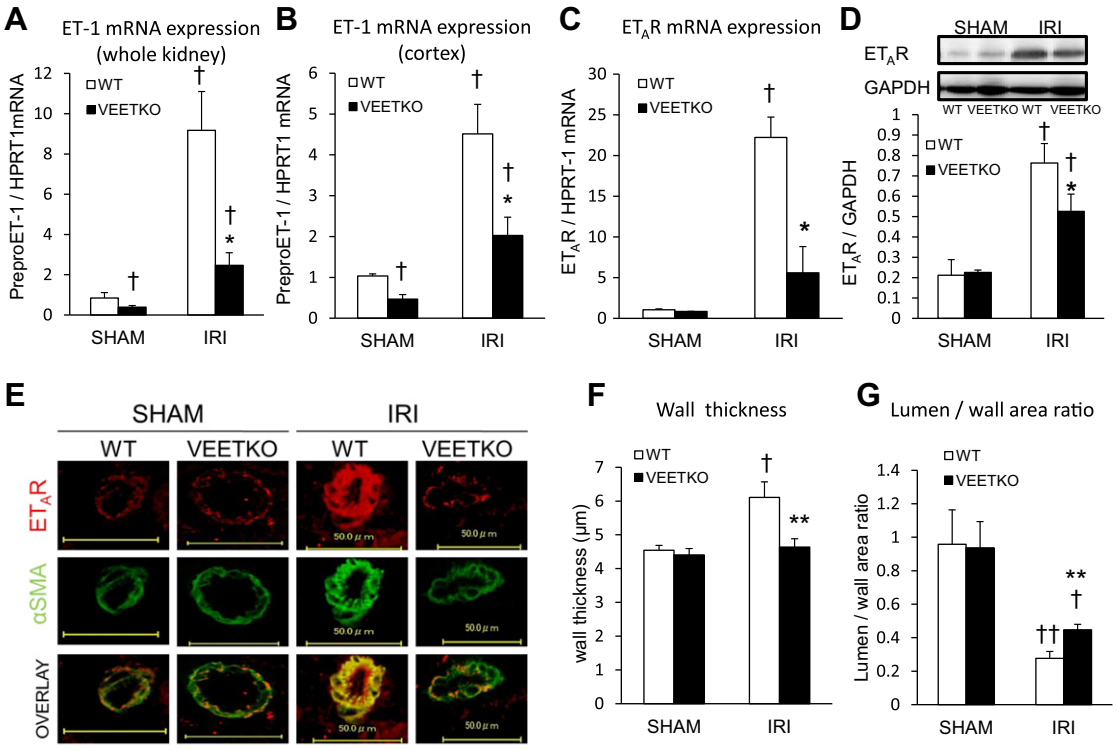

Fig. 2. ET-1 and ET_AR expression and arterial morphology after IRI. (A–E) Expression level of ET-1 and ET_AR after IRI was measured by real time PCR. (A) ET-1 mRNA expression in whole kidney and (B) in the cortex ($n = 4–5$). (C) ET_AR mRNA expression ($n = 4–5$). (D) Western blot and densitometric analysis of ET_AR expression ($n = 4$). (E–G) Wall thickness and ratio of lumen/wall area was calculated based on αSMA staining as described in the methods. (E) Representative images of αSMA and ET_AR staining in the kidney (F) Wall thickness and (G) ratio of lumen / wall area ($n = 4–5$). Results were expressed as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ versus WT IRI. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ versus WT sham. Bar = 50 μm.

higher than in WT mice (Fig. 2F and G). IF confirmed expression of ET_AR in vascular smooth muscle cells of intra-renal artery (Fig. 2E).

3.5. ROS production was reduced by ET-1 deletion from EC

ROS formation measured by DHE was increased after IRI. Quantification of DHE intensity revealed a significant reduction of DHE intensity in VEETKO mice after IRI compared to WT mice (Fig. 3A and B). 8-OHdG, an oxidative DNA damage marker extensively stained the nuclei of the epithelial cells, but not the interstitial cells in both genotypes after IRI (Fig. 3C). We observed a higher PKC protein abundance after IRI that was significantly reduced in VEETKO mice (Fig. 3D).

3.6. ET-1 deletion from EC reduced inflammatory response after IRI

Real time PCR analysis showed an increase of the renal cortical mRNA levels of inflammation mediators (MCP-1, ICAM-1, TLR2 and TLR4) after IRI (Fig. 4). These levels were lower in VEETKO mice compared to WT. There was no difference in F4/80, a macrophage marker, positive cells number between WT and VEETKO mice in SO groups. Dendritic cells in the interstitium were positive for F4/80. The number of F4/80 positive increased after IRI indicating that IRI induced profound infiltration of macrophage. F4/80 positive cells were lower in VEETKO mice compared to WT mice after IRI.

4. Discussion

This study showed that genetic suppression of ET-1 from the vascular endothelial cells improves renal function one day after IRI.

4.1. Effect of EC-ET-1 on vasoconstriction and hypoxia

The decrease of blood supply to the kidney is a major event of AKI. The elevation of kidney ET-1 24 h after AKI has been already reported [20]. Vasoconstriction effect of ET-1 in renal vascular system is mediated by ET_AR [21,22]. An increase of ET-1 is therefore proposed to further induce perfusion disturbance in the kidney after IRI [23]. Consistently, we showed an increase of ET-1 and ET_AR expression as well as a decrease of lumen/wall area ratio of small renal arterioles after IRI. This was prevented in VEETKO mice and may participate to a better renal perfusion after IRI. Moreover, ROS, which partly contribute to the vasoconstriction induced by ET_AR [24], were reduced in VEETKO after IRI compared to WT mice. In the renal cortex of VEETKO mice, eNOS protein abundance was higher than in WT after IRI (Supplementary Fig. 1). Vasoconstriction after IRI may be amplified in part by a reduction of NO because of endothelial cells damage [25]. NO inhibits vasoconstriction [26] and represents a counter-regulator of the ET-1 system [27]. The absence of EC-ET-1 may thus prevent vasoconstriction by maintaining eNOS protein abundance. Reduction of ET-1 and ET_AR activation in VEETKO mice thus possibly attenuates kidney injury through balancing renal vasoconstriction and vasodilatation.

Kidney IRI increased intra-renal artery wall thickness, which has been shown also after IRI in the focal cerebral ischemic model in rats [28]. IRI-induced increase of wall thickness was prevented in VEETKO mice indicating that EC-ET-1 is an important mediator of vascular dysfunction through its proliferative effects on smooth muscle cells. ET-1 has been already reported to act as a mitogen factor in small arteries *in vivo* [29]. *In vitro* studies showed that ET-1 induces proliferation of arterial smooth muscle cells [30,31]. In addition, treatment with bosentan, a non-selective endothelin antagonist, in hypertensive rat prevents vascular hypertrophy [32].

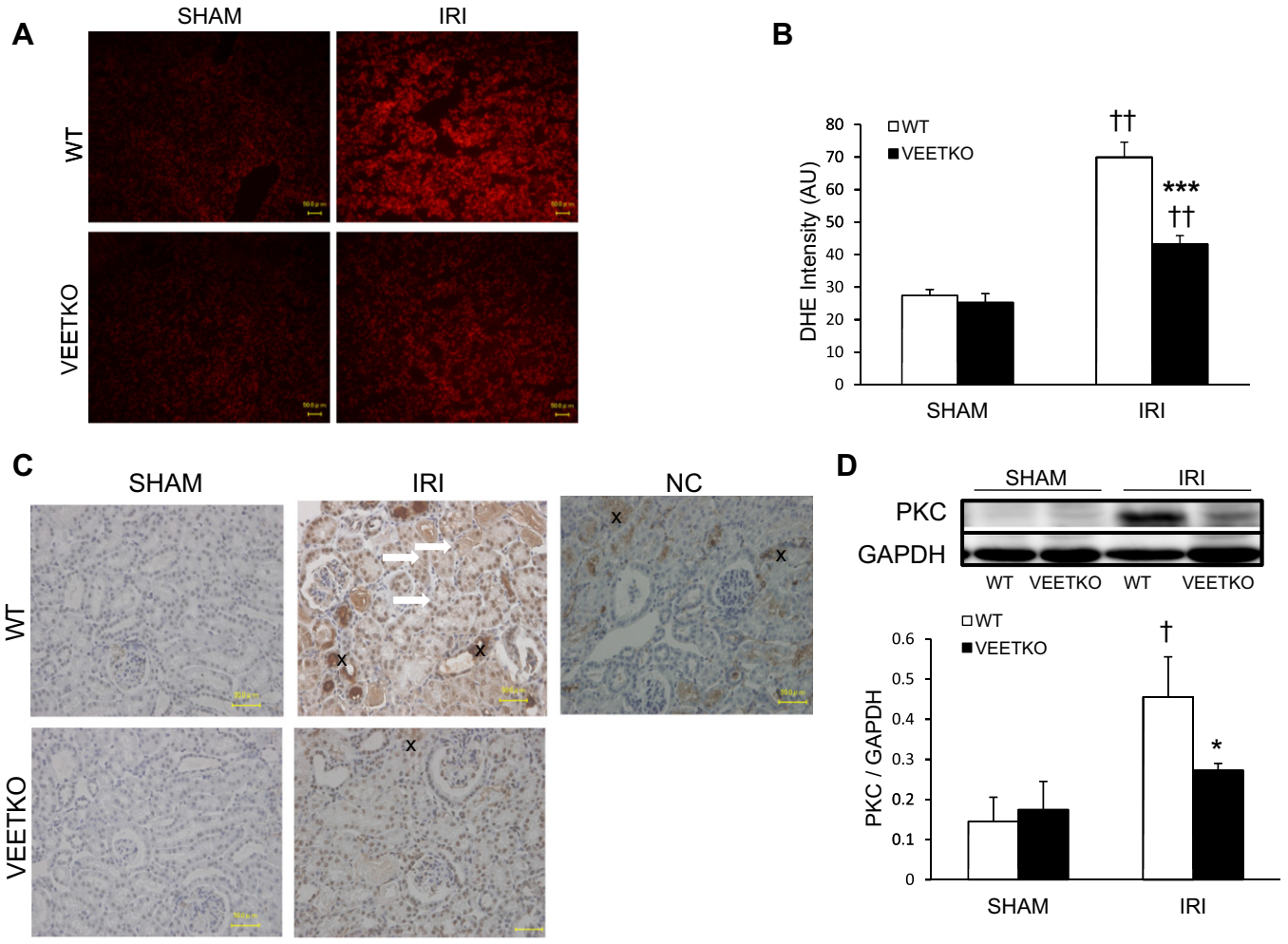

Fig. 3. Effect of IRI on PKC expression and Reactive Oxygen Species (ROS) production. (A–C) Oxidative stress was assessed by dihydroethidium (DHE) and 8OHdG staining. (A) Representative images of DHE staining (400× magnification, 12 fields each sample, $n = 5$). (B) Quantification of DHE signal. (C) Representative image of 8OHdG staining (arrow = nuclear staining). Staining in the intra-luminal cast (X) was due to unspecific binding of the secondary antibody. (D) Western blot and densitometric analysis of PKC ($n = 4$). $^{*}p < 0.05$, $^{***}P < 0.001$ versus WT IRI. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ versus WT sham. Bar = 50 μm.

Finally, we showed that in the cortex of WT mice after kidney IRI, tubular injury was more pronounced compared to the VEETKO mice. The increase of ET-1 expression in ischemic kidney occurs mainly in the peritubular capillaries [6]. On the other hand, immunohistological analysis indicate that the vascular endothelial cells are the main site of ET-1 expression in the cortex and ET-1 production in the renal cortex of VEETKO mice is restricted to a small portion of peritubular capillaries [17]. The hypoxia caused by the vasoconstriction mediated by ET-1 in these capillaries may have deleterious effects on the adjacent tubules [6]. Our results support this assumption by showing that the tubules are protected by the suppression of EC-ET-1.

4.2. Effect of EC-ET-1 on oxidative status

IRI generates excessive amount of ROS [33], which can be suppressed by the endothelin blocker bosentan [34]. Ischemia-induced increase in oxidative stress provokes endothelial dysfunction, which is mediated by the ET-1 dependent activation of PKC [35]. Our results indicated that inhibition of ET-1 from EC decreased ROS production (Fig. 3). Concomitantly, we observed a higher PKC protein abundance after IRI that was significantly reduced by ET-1 deletion from EC. ET-1 is known to activate PKC [35]. PKC can induce superoxide production and endothelium dysfunction in renovascular hypertension model in rat [36]. PKC may thus mediate ET-1–induced ROS production in kidney IRI. Finally, ROS induced DNA damage of epithelial cells, but not interstitial cells (Fig. 3C). This confirms previous observations showing that ROS affect epithelial cells exclusively but not interstitial cells after kidney IRI in mice [33].

4.3. Effect of EC-ET-1 on inflammation

Inflammation is one of the initial processes in the extension phase of IRI. Inflammation in IRI involves signaling events via patterns recognition molecules such as Toll-like receptors (TLR2, TLR4) and ICAM-1 [5,37,38]. Macrophages contribute to the early and late stages of renal IRI and appear in the kidney within 1–5 days followed by monocytes chemotactic protein [39]. In this study, ET-1 deletion from EC reduced inflammation responses in addition to prevent tubular injury. VEETKO mice presented significantly lower mRNA levels of TLR2, TLR4, ICAM-1 and MCP-1 as well as decreased macrophage cell number in the renal cortex (Fig. 4). It seems that reduction of the inflammatory response participated in the preservation of the tubular system in VEETKO mice after IRI. Our previous study using the blood flow cessation model by carotid ligation also showed reduction of inflammatory cells

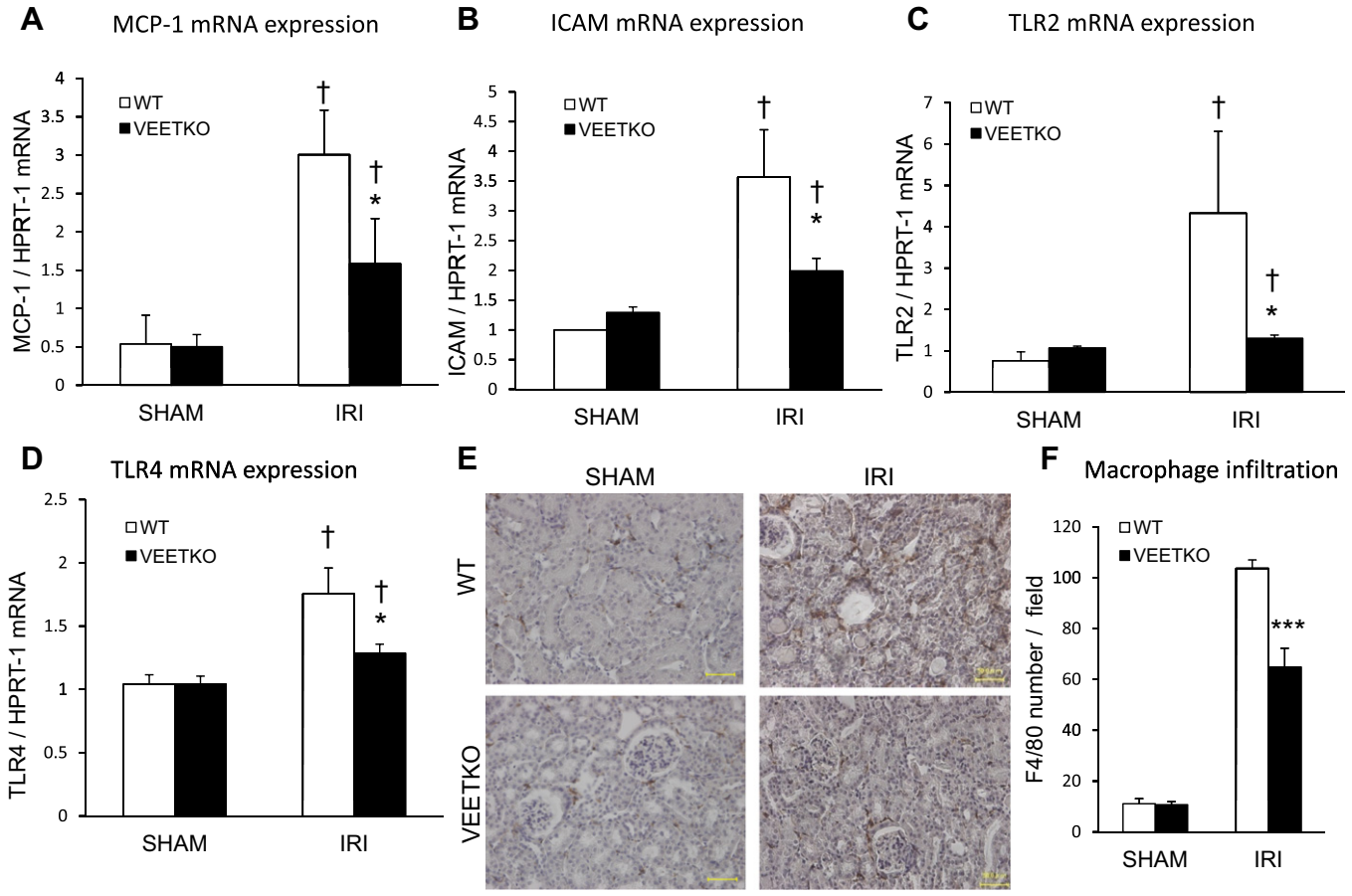

Fig. 4. Effect of ET-1 deletion from EC on the inflammatory process after IRI. (A–D) Inflammatory markers mRNA expression measured by real time PCR: (A) MCP-1, (B) ICAM-1, (C) TLR2 and (D) TLR4. (E and F) Macrophage infiltration was measured based on immunostaining of F4/80. (E) Representative images of F4/80 staining (400× magnification field, 12 fields each sample, $n = 5–6$). (F) Quantification of the number of F4/80 positive cells. Results were expressed as means ± SD. $^{*}p < 0.05$, $^{***}p \leqslant 0.001$ versus WT IRI. † $p \leqslant 0.05$ versus WT sham. Bar = 50 μm.

recruitment and inflammatory mediators in the vessels of the VEETKO mice compared to WT [40].

Taken together, ET-1 from EC may participate in the injury of tubular epithelial cells, and promote oxidative stress and inflammation during the extension phase of kidney IRI. Blocking ET-1 effects may provide new potential therapy in kidney IRI.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.121.

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