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

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(Degree)
博士（医学）

(Date of Degree)
2012-09-25

(Resource Type)
doctoral thesis

(Report Number)
甲5659

(URL)
https://hdl.handle.net/20.500.14094/D1005659

(課程博士関係)

学 位 論 文 の 内 容 要 旨

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

腎虚血再灌流モデルにおける内皮由来エンドセリン-1抑制がもたらす腎保護効果の解析

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1. INTRODUCTION

Acute kidney injury (AKI) is a common clinical problem with a high mortality rate, predominantly in intensive care units. Renal ischemia/reperfusion injury (IRI) represents the most frequent cause of AKI and leads to chronic progression in up to 70% of the cases.

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. Together with the hemodynamics changes, inflammation and tubular epithelial injury are major components of the pathophysiology of AKI. The role of the endothelial cells from the microvasculature in AKI is important, particularly in their ability to regulate inflammatory cell infiltration and tubular function.

Endothelin-1 (ET-1) that is mostly secreted by endothelial cells is a potent vasoconstrictor. ET-1 infusion in perfused kidney induces reduction of renal blood flow and glomerular filtration rate through its vasoconstriction effect. ET-1 is up-regulated in the renal ischemic period. 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. 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.

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. Four-month old male VEETKO mice and WT littermates (n=8) were subjected to kidney IRI. Briefly, mice were anesthetized using inhalational isoflurane. Abdomen was opened and both renal pedicles were clamped using non-traumatic microaneurysm clamps for 30 minutes. Sham operated (SO) mice (n=5) underwent similar procedures except for renal pedicles clamping. We sacrificed the mice on the day following the operation to examine the extension phase of kidney IRI. Renal tissues were used for RNA, protein and histology examination.

2.2. Kidney function assessment

Serum creatinine level was measured from blood collected from the orbital sinus.

2.3. Histological analysis

Periodic acid Schiff's reagent (PAS) was done to evaluate tubular injury. Immunohistochemical (IHC) staining was done for these following antibodies: F 4/80, PCNA and 8-OHdG/8-Hydroxy-2'-deoxyguanosine. Frozen sections were used for double immunofluorescence (IF) staining for alpha-smooth muscle actin / α SMA and ET_AR. DHE staining was done to examine ROS production after IRI. Vascular wall thickness was measured based on α SMA staining.

2.4. Real-time PCR and western blot

These following primers were used for real-time PCR quantification: ET-1, ET_AR, MCP-1, TLR2, TLR4, and ICAM-1. HPRT-1 (was used as reference. Western blot and densitometry analyses were done for these antibodies: E-Cadherin, eNOS, ET_AR, PKC and GAPDH antibody.

2.5. Statistics

Results were expressed as mean ± SD. 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 and cortex tubular injury after IRI

IRI induced renal failure as well as tubular injury as seen in elevation of creatine serum level and tubular injury score. VEETKO mice presented a significantly lower serum creatinine and tubular injury score level than WT mice. In WT mice, the injury spread to the cortex more extensively compare to VEETKO mice. PCNA immunostaining showed an increased positive signal in epithelial cells on day 1 after IRI. E-Cadherin expression decreased after IRI. ET-1 deletion from EC increased the number of PCNA positive epithelial cells and increased E-Cadherin protein abundance in renal tissue.

3.2. ET-1 and ET_AR expression increased after IRI and induced increase of vascular wall thickness

IRI induced ET-1 expression in whole kidney and cortex. Endothelial cell-derived ET-1 deletion significantly reduced mRNA ET-1 level. Similarly, IRI-induced increase of ET_AR

mRNA and ET_AR protein level was stronger in WT than in VEETKO mice. IF confirmed expression of ET_AR in vascular smooth muscle cells of intra-renal artery. Wall thickness increased in WT only after IRI. Lumen / wall area ratio decreased in both genotypes after IRI but was higher in VEETKO than in WT

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

ROS formation measured by DHE was increased after IRI. 8-OHdG, an oxidative DNA damage marker extensively stained the nuclei of the epithelial cells, but not the interstitial cells. Quantification of DHE intensity revealed a significant reduction of DHE intensity in VEETKO mice after IRI compared to WT mice.

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. These levels were lower in VEETKO mice compared to WT. IRI induced profound infiltration of macrophage. The number of F4/80 positive cells was lower in VEETKO mice cortex.

4. DISCUSSION

The elevation of kidney ET-1 24 hours after AKI has been already reported. An increase of ET-1 is therefore proposed to further induce perfusion disturbance in the kidney after IRI. 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, 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. Vasoconstriction after IRI may be amplified in part by a reduction of NO because of endothelial cells damage. NO inhibits vasoconstriction and represents a counter-regulator of the ET-1 system. Reduction of ET-1 and ET_AR activation in VEETKO mice thus possibly attenuates kidney injury through balancing renal vasoconstriction and vasodilatation.

IRI generates excessive amount of ROS, which can be suppressed by the endothelin blocker bosentan. Ischemia-induced increase in oxidative stress provokes endothelial dysfunction, which is mediated by the ET-1 dependent activation of PKC. Our results indicated that inhibition of ET-1 from EC decreased ROS production. 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. PKC induces superoxide production and endothelium dysfunction in renovascular hypertension model in rat. PKC may thus mediate ET-1 induced ROS production in kidney IRI.

Inflammation in IRI involves signaling events via patterns recognition molecules such as Toll-like receptors (TLR2, TLR4) and ICAM-1. 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. It seems that reduction of the inflammatory response participated in the preservation of the tubular system in VEETKO mice after IRI.

Taken together, this study showed that suppression of ET-1 from the vascular endothelial cells improves renal function on the day after IRI.

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論文審査の結果の要旨

受付番号	甲 第2290号	氏名	Nur Arfian
論文題目 Title of Dissertation	ET-1 deletion from endothelial cells protects the kidney during the extension phase of ishchemia/reperfusion injury 腎虚血再灌流モデルにおける内皮由来エンドセリン-1抑制がもたらす腎保護効果の解析		
審査委員 Examiner	主査 Chief Examiner 西 慎一 副査 Vice-examiner 的場 尚 副査 Vice-examiner 飯島 一誠		

(要旨は1，000字～2，000字程度)

急性腎障害を示す腎虚血再灌流障害においては、さまざまな因子が腎尿細管障害を惹起し、腎虚血再灌流障害の一部は慢性腎障害に進展する。この腎虚血再灌流障害の進行には、血管収縮因子と血管拡張因子のバラスンが関与している。また、同時に、炎症、酸化ストレスの亢進も関与していると言われる。

Endothelin-1(ET-1)は、血管内皮細胞から分泌される強力な血管収縮因子であり、また、炎症、酸化ストレスの亢進作用も有する多機能因子である。腎虚血再灌流障害においてこのET-1の生物学的機能を評価するために、ET-1 knock-out mouse(VEETKO mouse)を作成し多角的な検討を行った。

実験の方法としては、wild type mouse(WT mouse)を対照として、VEETKO mouseの双方に腎動脈を一時的にクランプして開放する腎虚血再灌流モデル(IRI モデル)を作製した。評価項目としては、腎機能、腎尿細管病理所見、腎内血管形態変化、そして、real-time PCR、western blotting、免疫組織化学法等を用いてET-1、ET-Aリセプター、αSMA、DHE、PKC、MCP-1、ICAM-1、TRL2、TRL4、F4/80、PCNAなどの発現量、組織学的局在などを評価した。

結果としては、VEETKO mouse IRIモデルの腎機能障害、尿細管障害病理所見はWT mouse IRI モデルより、軽度の障害にとどまっていた。VEETKO mouse IRI モデルでは、確かにET-1、ET-Aリセプターの発現が低下していた。また、WT IRI モデルでは腎内血管の血管壁肥厚と内腔狭窄が起こっていたが、VEETKO mouse IRI モデルではこれらの所見が軽減していた。血管壁平滑筋細胞において、ET-A、αSMA の発現が抑制されており、腎内血管壁肥厚と内腔狭窄の抑制は、これらの所見との関連が示唆された。また、酸化ストレスマーカーとしてDHE、PKCの発現を比較したが、やはりVEETKO mouse IRIモデルでその発現量が低下していた。MCP-1、ICAM-1、TRL2、TRL4、F4/80などの炎症関連マーカーも全て、VEETKO mouse IRIモデルでの発現が低下していることも確認された。

以上の結果より、ET-1は、IRI modelのAKIの進展に深く関与していることが実証された。特に、IRI modelでは、ET-1が腎内血管の内腔狭窄を促進し虚血障害を助長する可能性、酸化ストレスや炎症性サイトカイン、それらのリセプターの発現亢進、マクロファージ系細胞の浸潤などにも関与する可能性が明確に示された。IRIモデルのAKI進行機序にET-1が重要な役割を有しており、ET-1発現抑制あるいはET-1感受性抑制はAKI進行阻止に繋がることが示された。