Evaluation of neutrophil dynamics change by protective effect of tadalafil after renal ischemia/reperfusion using in vivo real-time imaging

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Authorship page

YMa, MA, and KK designed experiments; YM, KK, YS, KY and YMi performed experiments; YMa, MA, KK wrote the article; TS, KW, MW, TM and MS analyzed data and revised the articles; NK and YN approved the article for submission.

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Abbreviations Page

eNOS, endothelial NOS

eNOS-KO, eNOS knockout

IRI, ischemia/reperfusion injury

KIM-1, kidney injury molecule 1

NO, nitric oxide

NOS, nitric oxide synthase

PAS, periodic acid-Schiff

PDE, phosphodiesterase

SEM, standard error of the mean

ROS, reactive oxygen species

WT, Wild-type

Abstract

Background. Neutrophils play a major role in ischemia/reperfusion injury (IRI) in renal transplantation and acute kidney injury. However, it has been difficult to observe changes in neutrophil dynamics over time in living mice kidney. We investigate neutrophil dynamics in IRI in living mice using novel *in vivo* multiphoton microscope imaging techniques and characterize the renoprotective effects of a selective phosphodiesterase (PDE) 5 inhibitor, tadalafil.

Methods. Wild-type (WT) and eNOS knockout (eNOS-KO) mice, a model of endothelial dysfunction, were used to establish *in vivo* real-time imaging in living mouse kidneys. Neutrophils were labeled green with Ly-6G monoclonal antibody, and plasma flow was labeled red with bovine serum albumin. Tadalafil was administered orally 1 h before surgery. Both kidney pedicles were reperfused after 37℃ warm ischemia for 45 min.

Results. Our novel approach revealed that neutrophils were trapped in glomerulus within a few minutes after reperfusion. They gradually increased over time and Infiltrated neutrophils were observed in the tubular lumen and peritubular capillary. The neutrophils were clearly visualized rolling on peritubular capillary plexus at 3 μm/min. The administration of tadalafil significantly reduced

neutrophil influx into the glomerulus in both WT and eNOS-KO mice. Reduced neutrophil infiltration in tadalafil groups, which was confirmed by flow cytometry, resulted in histopathologically decreased tubular injury. The expression of VCAM-1 and KIM-1 was partially prevented by tadalafil.

Conclusions. Use of a novel technique contributed to elucidation of neutrophil dynamics after reperfusion. Tadalafil has a potential for inhibiting neutrophil infiltration in renal IRI.

Introduction

Ischemia/reperfusion injury (IRI) is an unavoidable consequence of solid organ transplantation, and has a critical impact on the functional outcome of allografts.[1,2] For renal allografts, IRI induces a strong inflammatory response that promotes apoptosis of tubular epithelial cells and vascular endothelial cells.[3-5] Key processes associated with this inflammatory response include neutrophil infiltration and activation in the ischemic vascular endothelium.[5-8] Within minutes of organ reperfusion, neutrophil chemoattractants, including IL-8, CXCL1/KC and CXCL2/MIP-2, are produced in the ischemic tissue.[9,10] The neutrophils play a major role in tissue injury through the release of cytokines, tissue degrading enzymes and reactive oxygen species (ROS), and early post-transplant neutrophil infiltration is negatively associated with short- and long-term graft function.[10,11] Therefore, elucidation of neutrophil dynamics immediately after reperfusion is crucial for exploring the diagnosis and treatment of renal IRI.

A major problem in the study of neutrophil dynamics is that mice must be sacrificed for observation of developing pathology. The changes in renal tissue over time cannot be seen in real time, thus the tissue assessment is conducted in a different individual mouse at each time point. In addition, the various

processes in the preparation of pathological specimens, such as fixation or washing, may have interfered with the correct evaluation. Thus, previous observation of neutrophils has deviated from actual dynamics. To solve these problems, in the present study, we established *in vivo* real-time imaging of neutrophils in living mouse kidneys using multiphoton microscope. Our technique allows to analysis the changes in neutrophil dynamics in the same living mouse kidneys after ischemia/reperfusion over time.

Studies in several animal models have shown that attenuation of neutrophil infiltration results in decreased tissue injury during reperfusion of ischemic tissues.[6,12,13] The protection of tubular epithelium and endothelial cells prevents the production of neutrophil chemoattractants and endothelial cell-neutrophil adhesion.[14] Tadalafil, a selective phosphodiesterase (PDE) 5 inhibitor, is commonly used for pharmacological therapy in cardiovascular diseases and erectile dysfunction. PDE5 isozymes catalyzes the hydrolysis of cyclic guanosine monophosphate (cGMP).[15] cGMP activates protein kinase G, causing a decrease in intracellular calcium concentration, which leads to relaxation of vascular smooth muscle cells.[16] Inhibition of PDE5 with tadalafil increases intracellular levels of cGMP and promotes vasodilation, resulting in improved endothelial

function.[16,17] Several studies have revealed the tissue-protective effects by PDE5 inhibitors against ischemic injury including renal IRI.[17-22] However, its mechanism is still unclear.

NO is produced by endothelial NOS (eNOS) plays an important role in the maintenance of vascular function, including regulation of adhesion molecules.[23,24] Furthermore, eNOS has a pivotal protective role in renal IRI by relaxing blood vessels and preserving renal blood flow.[23,25,26] Thus, endothelial dysfunction which caused by impaired eNOS reduces the tolerance to ischemia and affects the survival rate of allografts. These endothelial dysfunction is a common pathophysiologic mechanism in both diabetic vascular complications and aging.[27-30] However, neutrophil dynamics after allografting or tissue-protective effects of PED5 inhibitor in the presence of endothelial dysfunction have not been fully elucidated.

In this study, we developed the novel *in vivo* imaging techniques assessing neutrophil dynamics, and investigated the renoprotective effects of PDE5 inhibitor by focusing the impact on neutrophil infiltration in a mouse IRI model.

MATERIALS AND METHODS

Animals and renal ischemia/reperfusion

Six- to ten-week-old male wild-type (WT) and eNOS knockout (eNOS-KO) mice weighing 20-30 g were used throughout the study. WT and eNOS-KO mice were purchased from Jackson Laboratory (Bar Harbor, ME).[31] All mice had the same C57BL/6J background. Mice were housed in a temperature- and humidity-controlled room with a 12-h light-dark cycle.

To detect the effects of tadalafil, WT and eNOS-KO mice were randomly divided into 3 groups: control, ischemia/reperfusion without tadalafil (IRI) and ischemia/reperfusion with tadalafil (IRI + PDE5). Tadalafil (0.2 mL; 0.5 mg/mL) was administered orally 1 h before surgery for the IRI + PDE5 group, and saline (0.2 mL) was administered to the control group and IRI group. The mice were anaesthetized with medetomidine, midazolam and butorphanol and maintained their rectal temperature at 37℃ using a homeothermic blanket system (Bio Research Center Co, Ltd, Nagoya, Japan) during procedures. Both kidney pedicles were identified through a flank incision, and clamped for 45 min. Control animals were subjected to the same surgical procedure without having their kidney pedicles clamped. The clamps were released for reperfusion. Color

changes of kidneys were monitored to indicate sufficient renal ischemia/reperfusion during the initiation of clamping and after removal of clamps. Kidney samples and blood were collected after the indicated durations of reperfusion for the following studies.

Multiphoton *in vivo* imaging

The mice were anaesthetized with sevoflurane. The left kidney was exposed through a flank incision prior to placing the mice on a microscope stage. The images were acquired using a Nikon AIR-MP multiphoton confocal microscope (Tokyo, Japan) equipped with an inverted imaging system and Apo LWD25X 1.10W DIC N2 objective lens. Alexa Fluor 594-conjugated bovine serum albumin (A13101) was injected to label the plasma flow (100 μL of a 5 mg/mL stock, i.v. bolus, Invitrogen, Japan). To label the neutrophils, anti-mouse Ly-6G monoclonal antibody (RB6-8C5; Bio X Cell, Tokyo, Japan), conjugated with Alexa Fluor 488-Lightning-Link Rapid Conjugation System (Innova Biosciences), was injected as a bolus (40 μg). Multiphoton imaging was used with 800-nm laser excitation and detection of bovine serum albumin, Alexa Fluor 594 conjugate through 595/50 nm bandpass filters and anti-mouse Ly-6G monoclonal antibody, Alexa Fluor 488

conjugate through 525/50 nm bandpass filters. To avoid the potential laser toxicity, laser excitation was minimized by using a low laser power. 1.5 μm Z-stack images with a thickness of about 40μm were taken of all observable glomeruli. No apparent cell injury was observed due to short-time image acquisition of 1 Z-stack per glomerulus, less than 2minutes. The left kidneys were subjected to ischemia for 45 minutes. After reperfusion, the observation was continued up to 4 h. The rectal temperature was also monitored and maintained at 37°C using a homeothermic blanket system (Bio Research). To prevent dehydration, 0.2 mL of saline was injected into the peritoneal cavity before reperfusion and after every 30 min. The percentage of neutrophils in the glomerulus was calculated from the ratio of the volume of green fluorescent positive area to the volume of the glomerulus, using Nikon imaging software NIS Elements Advanced Research. After setting the ROI (region of interest) to surround the glomerulus, thresholding of Alexa594 (red, BSA) and Alexa 488 (green, Ly-6G) was performed manually from binary image in every slice of 3D volume imaging. Then, the glomerular volume and the volume of Alexa 488-positive area were measured by the volume measurement analysis.

Histology and immunohistochemistry

Kidney tissue was fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections (4 μm thick) were deparaffinized and treated for periodic acid-Schiff (PAS) staining. For immunochemical staining, kidney sections (4 μm thick) were rehydrated and subjected to antigen retrieval in a microwave, followed by incubating overnight with an antibody against kidney injury molecule 1 (KIM-1) (KCA031610A, R&D Systems, Minneapolis, MN, USA). The primary antibody was detected using the Histofine Simple Stain MAX-PO kit (Nichirei Corporation, Tokyo, Japan) and 3,3'-diaminobenzidine (Sigma-Aldrich; Merck KGaA).

The tubular damage was examined by PAS staining in a blind manner and evaluated using the following scoring system based upon the percentage of damaged tubules: 0, no damage; 1, <25%; 2, 25 to 50%; 3, 50 to 75%; 4, >75%. The severity of tubular injury was also evaluated by examining 10 optional fields in randomly selected tissue samples. The images of areas with positive staining for KIM-1 were quantified using a color image analyzer (KEYENCE). The glomeruli and blood vessels of the cortex were excluded. KIM-1-positive areas were measured, and the results were presented as percentage of the relative volume of the scanned intersitium.[2]

Flow cytometric analysis

Kidney cells were prepared according to a protocol described previously.[19] Kidney tissue was homogenized using gentleMACS™ (Miltenyi Biotec, Tokyo, Japan) with collagenase type Ⅰ (1 mg/mL, Sigma Aldrich) and DNase Ⅰ (0.1 mg/mL, Sigma Aldrich) containing Hanks' buffered saline solution. The cells were dissolved in FACS buffer, and after 3 washing steps, incubated with a combination of the following directly conjugated antibodies: allophycocyanin-conjugated CD45, phycoerythrin-conjugated CD11b, Brilliant Violet 421™-conjugated Ly-6G and 7-AAD (all from BioLegend, San Diego, CA, USA). FACS Canto Ⅱ™ (BD Bioscience, Tokyo, Japan) and FlowJo software (Tree Star, Ashland, OR, USA) were used to perform flow cytometric analysis. Cell sorting was performed with a FACSAria™ Ⅲ (BD Bioscience) prior to RNA extraction using an RNeasy Kit (Qiagen, Germantown, MD, USA).

RNA extraction and Real-time quantitative PCR

Total RNA was isolated from the kidney tissue with TRIzol (Invitrogen Japan, Tokyo, Japan). Reverse transcriptase reactions were performed using a Ready-

To-Go T-Primed First Strand Kit (GE Healthcare Bio-science, Tokyo, Japan) for first strand cDNA synthesis. Real-time quantitative PCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers and probes for TaqMan analysis were designed using sequence information from GenBank (National Institutes of Health, Bethesda, MD, USA) and Primer3 online software (http://frodo.wi.mit.edu/primer3/; accessed July 1, 2015). The primers and probe sequence for mouse ICAM-1 were as follows: 5'- agctagcggaccagatcct -3' (forward primer); 5'- cagttacttggctcccttcc -3' (reverse primer); and 5'-FAM-ccctgagccagctggaggtctc -TAMRA-3' (TaqMan probe). Primer and probe sequences for mouse VCAM-1 were as follows: 5'- cccaaggatccagagattca-3' (forward primer); 5'-tgtctggagccaaacacttg-3' (reverse primer); and 5'-FAM-attcagtggccccctggaggtt -TAMRA-3' (TaqMan probe).

The level of mRNA expression in each sample was quantified using the absolute quantification standard curve method. The plasmid cDNA of each gene was used to prepare the absolute standards. The concentration was measured using the A260, which was converted to the number of copies using the molecular weight of the DNA. Each mRNA expression level was normalized to that of the

housekeeping 18S ribosomal RNA gene.

Statistical analysis

All values are expressed as the mean ± standard error of the mean (SEM). Statistical comparisons were performed using the two-tailed unpaired Student's *t*-test and one-way ANOVA, as appropriate. $P<0.05$ was considered statistically significant for all analyses. All statistical analyses were performed using EZR version 1.36 (Saitama Medical Center, Jichi Medical University), a graphical user interface for R.[32]

RESULTS

Neutrophil infiltration assessed by multiphoton *in vivo* imaging

To elucidate neutrophil dynamics in the kidney immediately after reperfusion, we first performed *in vivo* real time imaging using multiphoton microscopy. The multiphoton *in vivo* imaging of neutrophils revealed several new findings. First, immediately after IRI, blood flow returned to the kidney tissue, and the collapsed glomerulus bulged (Supplemental video 1). The diameter of the glomerulus expanded from 40μm to 60μm at the time of reperfusion. The leakage of albumin (BSA-Alexa594) was also observed in the urinary space and tubular lumen. A representative serial images before and after IRI is shown in Figure 1a. Within a few minutes of the beginning of reperfusion, neutrophils began to be trapped in the glomerulus and gradually increased over time. We were able to observe the infiltration of neutrophils into the glomerulus from afferent arterioles (Figure 1b and Supplemental video 2). After Neutrophils were trapped within the glomerulus, we were able to observe neutrophils in the tubular lumen and peritubular capillary. (Figure 1c), and they are identified 3 h post-reperfusion in most of the groups. After 24 hours, we also could observe neutrophils infiltrating inside and outside of peritubular capillary (Figure 1d). In addition, we succeeded in capturing

neutrophils rolling in the peritubular capillaries 24 h after renal IRI in WT mice (Supplemental video 3). The neutrophils that were most visible in the video moved about 3μm/min (30 min of continuous shooting was compressed to 6 sec).

Histological examination

We used PAS staining of kidney tissue samples 24 h after renal IRI to assess tubulointerstitial injury (Figure 2a). In both WT and eNOS-KO mice, the tubular injury score was significantly higher in the IRI groups than in the IRI + PDE5 groups (p=0.036. p=0.038 respectively) (Figure 2b).

FACS analysis of neutrophils in whole kidney tissues

To confirm tadalafil's efficacy in preventing neutrophil infiltration, we assessed neutrophils accumulation in whole kidney tissue using flow cytometry. In both WT and eNOS-KO mice, renal IRI induced accumulation of CD45+ CD11b+ Ly6G+ living cells compared to control group (Figure 3). The neutrophil (CD45+ CD11b+ Ly6G+) fraction was significantly lower in IRI + PDE5 groups compared to IRI groups 3 h after renal IRI (WT; p=0.035, eNOS-KO; p=0.044).

Multiphoton real-time imaging of tissue in vivo

Multiphoton *in vivo* real-time imaging was performed to confirm the neutrophil infiltration at more localized area in living mice kidney including the glomerulus and tubules. After reperfusion, neutrophils were trapped in the glomerulus, and neutrophils flowed out to the tubules a few hours later in both groups. Administration of tadalafil reduced neutrophil influx into the glomerulus and outflow to the tubules (Figure 4a). The percentage of neutrophils in the glomerulus 1 h after reperfusion was compared. Neutrophil ratio was significantly higher in eNOS-KO IRI group than WT IRI group ($p=0.043$), and significantly decreased by tadalafil in both WT ($p=0.046$) and eNOS-KO ($p=0.011$) mice.

Expression of ICAM-1 and VCAM-1 mRNAs assessed by real-time PCR in the kidney

Expression of ICAM-1 and VCAM-1 mRNAs 3 h after reperfusion was assessed by real-time PCR (Figure 5a and 5b). The expression of ICAM-1 and VCAM-1 mRNAs was increased in all of the IRI groups compared with the control group. The expression of VCAM-1 was significantly decreased by administering tadalafil in eNOS-KO mice ($p=0.017$). ICAM-1 and VCAM-1 mRNAs had no significant difference between WT IRI group and WT PDE5 group. There was no significant

difference in ICAM-1 and VCAM-1 between WT and eNOS-KO mice..

Tubulointerstitial damage assessed by KIM-1 staining

Tubular injury was evaluated by KIM-1 immunostaining (Figure 6a). The KIM-1-positive area was significantly larger in the eNOS KO IRI group than in the eNOS-KO-IRI + PDE5 group ($p=0.010$) (Figure 6b), however, the difference did not reach statistical significance. The difference between the WT-IRI group and the WT IRI + PDE5 group did not reach statistical significance.

DISCUSSION

In this study, we conducted *in vivo* real-time imaging of neutrophil dynamics after renal IRI using direct, *in vivo* visualization techniques. To our knowledge, this is the first report that captures the real-time changes of neutrophils and measures the speed of neutrophils rolling in a living mouse kidney by employing multiphoton laser microscopy. This approach allows us to capture the dynamic migration of neutrophils in the same living mice kidney without sacrifice. Furthermore, using this novel approach, we confirmed that tadalafil inhibits neutrophil infiltration into glomerular in renal IRI, even under condition of eNOS deficiency.

The novel in vivo real-time imaging of neutrophil dynamics

Renal IRI is a main cause of episodes of delayed graft function or increased rates of acute rejection.[1,2] Previous studies have shown that neutrophils quickly infiltrate ischemic tissues immediately after reperfusion.[5-8] Infiltrating neutrophils mediate oxidant stress and release granules containing proteolytic enzymes resulting in tissue damage.[33,34] However, neutrophil dynamics immediately after renal IRI have remained unclear due to the technical difficulties of visualizing neutrophil recruitment.[35] Our innovative imaging approach revealed that

neutrophils were initially trapped in the glomerulus after it bulged and then collapsed upon resumption of blood flow. Subsequently, we were able to observe neutrophils infiltrating the tubular lumen and peritubular capillary. Previous reports of neutrophil immunostaining in renal IRI indicated that neutrophils accumulated around the renal tubules at the cortico-medullary junction due to vulnerability to ischemia.[6,36] In this study, most of the neutrophils trapped in the glomerulus were transported to the ischemic tissue. The various processes in the preparation of pathological specimens, such as fixation or washing, may have interfered with the correct evaluation in previous studies. In multiphoton laser microscopy, it is difficult to observe the depth of the cortico-medullary region; however, in observations 24 hours after reperfusion, neutrophils were seen infiltrating around the renal tubules as previous pathological studies have reported.[37,38] We also observed neutrophil rolling in the peritubular capillary plexus 24 hours after IRI *in vivo.* Therefore, we were able to measure the speed of neutrophil rolling. To our knowledge, this is the first report of neutrophil *in vivo* rolling video footage and measurement of its speed. Neutrophils recruited to the cortical vessels of the kidney are captured by selectin, and they transition from rolling to slow rolling by the binding to ICAM-1 on endothelial cells.[39,40] The

visualization of neutrophil recruitment into the kidneys after reperfusion will contribute to further analysis of the dynamics. In addition, using our technique, it may be possible to observe additional leucocytes in the kidney, including macrophages. The major problem in previous studies of neutrophil dynamics has been the necessity of sacrificing the mice at every time point. Our novel technique avoided that problem and thereby reduces the loss of experimental animals.

The protective effects of tadalafil on renal ischemia reperfusion injury

Endothelial dysfunction is a common vascular condition in diabetics and the elderly. It is a critical factor affecting the survival outcomes of allografting. Since the efficacy of tadalafil in patients with endothelial dysfunction is still unclear, we investigated its efficacy using eNOS-KO mice in this study. Tadalafil impacts the rate-limiting enzymes for cGMP-induced vasodilation and provides an anti-inflammatory effect by increasing the levels of cGMP and NO during renal IRI.[17,20,41] In the kidney, PDE5 is localized to the glomeruli, cortical tubules, vasculature, mesangial cells and inner medullary collecting duct. Tadalafil has a pharmacodynamic profile that maintains vasodilatory action for up to 36 h and its half-life is 4-times longer (17.5 h) than sildenafil, which was the first PDE5

inhibitor to be evaluated as a protectant during renal IRI.[42-45] Since the vasodialatory effect of PDE5 inhibitor is closely related to the NO/cGMP pathway, it has been reported that the effect of PDE5 inhibitor disappears under eNOS deficiency. However, there is some reports that PDE5 inhibitor markedly showed protective effects in eNOS-deficient mice, thus it is controversial to conclude.[46,47] In this study, histopathological observation showed that tadalafil decreased tubular injury in kidney tissue after reperfusion. Neutrophils, main cause of tissue injury, were reduced by tadalafil in flow cytometry using whole kidney tissue, however the differences between WT and eNOS-KO mice did not reach statistical significance. In multiphoton real-time imaging, neutrophil accumulation was also reduced by tadalafil in both WT and eNOS-KO groups, and was significantly higher in eNOS-KO mice than WT mice. These differences were detected because flow cytometry analyzed the whole kidney tissue, whereas real-time imaging can focus on a more localized area which is the glomerulus. The protective effects of tadalafil are mediated by intracellular cGMP levels as well as the activation of mitochondrial K-ATP channels either directly or indirectly through various signaling pathways, such as activation of protein kinase G or protein kinase C.[48] The increased cGMP level stimulates soluble guanylate cyclase,

which plays an important role in the synthesis of NO, and improves the impaired renal vasodilator response to exogenous NO in kidneys.[49,50] This mechanism of tadalafil may be effective even in eNOS-deficient conditions by elevating other NOS isforms expression.[51] In addition, several reports suggest PDE5 inhibitor can cause protective effects by a mechanism independent of the NO/cGMP pathway.[47,52,53]

Neutrophil infiltration into the renal interstitium

Neutrophils generally attach to endothelium and infiltrate into the kidney through their binding to ICAM-1 and VCAM-1.[11] The ROS induces adhesion molecules in renal endothelial cells, and the expression of ICAM-1 and VCAM-1 is regulated by ROS-dependent NF-kB activation.[54,55] The renal IRI produces ROS resulting in the increased expression of ICAM-1 and VCAM-1. We showed that tadalafil reduced the accumulation of neutrophils in the kidney by suppressing VCAM-1 expression in eNOS-KO mice. The ICAM-1 expression was also suppressed by tadalafil in eNOS-KO mice, although it was not significant. These suppression was not observed in WT mice. These results suggest that suppression of ICAM-1 and VCAM-1 by tadalafil is subtle, but it was augmented in endothelial

dysfunction model, eNOS-KO. In addition, our findings using real-time imaging showed that neutrophils were trapped in the glomerulus and flowed out into the tubules, suggesting that a mechanism of non-vascular neutrophil transmigration to the interstitial compartment complemented neutrophil transmigration through the vascular wall. The existence of neutrophil populations crossing the kidney interstitial compartment and tubular lumen had been confirmed by immunohistochemical localization in another report.[56] We used whole kidney to investigate the expression of ICAM-1 and VCAM-1. Clearer difference might be obtained by using an isolated glomerulus.

Despite the strength of direct observation of neutrophil dynamics, there are several limitations to this study. One is that it has not been verified that the ischemic time and rectal temperature were optimal for assessing differences among the groups. The extent of renal fibrosis and ischemic injury are highly influenced by increasing body temperature during ischemia.[57] Second, long-term observations over 24 h were not explored in this study. Third, whereas we successfully captured neutrophil dynamics, the observation of the cortico-medullary junction was limited to the superficial area due to technical difficulties.

In conclusion, for the first time, we directly visualized neutrophil dynamics in

living mouse kidney after IRI using multiphoton laser microscopy. This novel approach to *in vivo* imaging will contribute to the elucidation of neutrophil dynamics after reperfusion. Moreover, tadalafil reduced the accumulation of neutrophils in the glomerulus after reperfusion in both WT and eNOS-KO mice. Clinically, our study suggests that administration of tadalafil before transplantation will be useful to attenuate tissue damage during ischemia and kidney transplantation.

Supplemental data

Supplementary data are available at online.

Study approval

All animal experiments were conducted with adherence to the National Institutes of Health Guide for Care and Use of Laboratory Animals. The animal protocol was approved by Okayama University Animal Research Committee (approval no. OKU-2017075) and the Animal Research Committee of Kawasaki Medical School (approval no.18-102).

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REFERENCES

1. Nakayama T, Sato W, Kosugi T, et al. Endothelial injury due to eNOS deficiency accelerates the progression of chronic renal disease in the mouse. *Am J Physiol Renal Physiol.* 2009;**296**:F317-27.
2. Nishi Y, Satoh M, Nagasu H, et al. Selective estrogen receptor modulation attenuates proteinuria-induced renal tubular damage by modulating mitochondrial oxidative status. *Kidney Int*. 2013;**83**:662-673.
3. Jang HR and Rabb H. The innate immune response in ischemic acute kidney injury. *Clin Immunol*. 2009;**130**:41-50.
4. Basile DP. The endothelial cell in ischemic acute kidney injury: implications for acute and chronic function. *Kidney Int.* 2007;**72**:151-156.
5. Raup-Konsavage WM, Wang Y, Wang WW, Feliers D, Ruan H, Reeves WB. Neutrophil peptidyl arginine deiminase-4 had a pivotal role in ischemia/reperfusion-induced acute kidney injury. *Kidney Int*. 2018;**93**:365-374.
6. Miura M, Fu X, Zhang QW, Remick DG, Fairchild RL. Neutralization of Gro alpha and macrophage inflammatory protein-2 attenuates renal ischemia/reperfusion injury. *Am J Pathol*. 2001;**159**:2137-2145.

7. Linas SL, Shanley PF, Whittenburg D, Berger E, Repine JE. Neutrophils accentuate ischemia-reperfusion injury in isolated perfused rat kidneys. *Am J Physiol*. 1988;**255**:F726-735.
8. Rabb H, O'Meara YM, Madema P, Coleman P, Brady HR. Leukocytes, cell adhesion molecules and ischemic acute renal failure. *Kidney Int*. 1997;**51**:1463-1468.
9. Morita K, Miura M, Paolone DR, et al. Early chemokine cascades in murine cardiac grafts regulate T cell recruitment and progression of acute allograft rejection. *J Immunol*. 2001;**167**:2979-2984.
10. Araki M, Fahmy N, Zhou L, et al. Expression of IL-8 during reperfusion of renal allografts is dependent on ischemic time. *Transplantation*. 2006;**81**:783-788.
11. Kulkarni U, Goldstein DR. Divergent Roles for Neutrophils: Promoting and Resolving Inflammation? *Transplantation.* 2018;**102**:542-543.
12. Cugini D, Azzollini N, Gagliardini E, et al. Inhibition of the chemokine receptor CXCR2 prevents kidney graft function deterioration due to ischemia/reperfusion. *Kidney Int*. 2005;**67**:1753-1761.
13. Seekamp A, Mulligan MS, Till GO, Ward PA. Requirements for neutrophil

products and L-arginine in ischemia-reperfusion injury. *Am J Pathol.* 1993;**142**:1217-1226.

14. Bonventre JV, Zuk A. Ischemic acute renal failure: an inflammatory disease? *Kidney Int*. 2004;**66**:480-485.
15. McAuley IW, Kim NN, Goldstein I, Traish AM. Intracavernosal sildenafil facilitates penile erection independent of the nitric oxide pathway. *J Androl*. 2001;**22**:623-628.
16. Mazo E, Gamidov S, Iremashvili V. The effect of vardenafil on endothelial function of brachial and cavernous arteries. *Int J Impot Res*. 2006;**18**:464-469.
17. Liu F, Ni W, Zhang J, Wang G, Li F, Ren W. Administration of Curcumin Protects Kidney Tubules Against Renal Ischemia-Reperfusion Injury (RIRI) by Modulating Nitric Oxide (NO) Signaling Pathway. *Cell Physiol Biochem*. 2017;**44**:401-411.
18. Thieme M, Sivritas SH, Mergia E, et al. Phosphodiesterase 5 inhibition ameliorates angiotensin II-dependent hypertension and renal vascular dysfunction. *Am J Physiol Renal Physiol.* 2017;**312**:F474-481.
19. Kukreja RC, Salloum F, Das A, et al. Pharmacological preconditioning with

sildenafil: Basic mechanisms and clinical implications. *Vascul Pharmacol.* 2005;**42**:219-232.

20. Ahmad N, Wang Y, Ali AK, Ashraf M. Long-acting phosphodiesterase-5 inhibitor, tadalafil, induces sustained cardioprotection against lethal ischemic injury. *Am J Physiol Heart Circ Physiol*. 2009;**297**:H387-391.
21. Gasanov F, Aytac B, Vuruskan H. The effects of tadalafil on renal ischemia reperfusion injury: an experimental study. *Bosn J Basic Med Sci*. 2011;**11**:158-162.
22. Wietzikoski EGG, Foiatto JC, Czeczko NG, et al. Tadalafil protector effect during ischemia-reperfusion in rats. *Acta Cir Bras*. 2017;**32:**973-983.
23. Yamasowa H, Shimizu S, Inoue T, Takaoka M, Matsumura Y. Endothelial nitric oxide contributes to the renal protective effects of ischemic preconditioning. *J Pharmacol Exp Ther*. 2005;**312**:153-159.
24. Oztürk H, Dokucu AI, Buyukbayram H. Interaction of nitric oxide and cell adhesion molecules after 24 hours of complete ureteric obstruction in the rats on a solitary kidney. *Int Urol Nephrol*. 2001;**33**:435-443.
25. Förstermann U, Sessa WC. Nitric oxide synthases: regulation and function. *Eur Heart J*. 2012;**33**:829-837.

26. Gholampour H, Moezi L, Shafaroodi H. Aripiprazole prevents renal ischemia/reperfusion injury in rats, probably through nitric oxide involvement. *Eur J Pharmacol*. 2017;**813**:17-23.

27. Stehouwer CD, Lambert J, Donker AJ, van Hinsbergh VW. Endothelial dysfunction and pathogenesis of diabetic angiopathy. *Cardiovasc Res*. 1997;34:55-68.

28. De Caterina R. Endothelial dysfunction and pathogenesis of diabetic angiopathy. *Curr Opin Lipidol*. 2000;**11**:9-23.

29. Rischen-Vos J, van der Woude FJ, Tegzess AM, et al. Increased morbidity and mortality in patients with diabetes mellitus after kidney transplantation as compared with non-diabetic patients. *Nephrol Dial Transplan*t. 1992;**7**:433-437.

30. Neri F1 Furian L, Cavallin F, et al. How does age affect the outcome of kidney transplantation in elderly recipients? *Clin Transplant*. 2017;**31**. doi: 10.1111/ctr.13036. Epub 2017 Aug 20.

31. Sogawa Y, Nagasu H, Iwase S, et al. Infiltration of M1, but not M2, macrophages is impaired after unilateral ureter obstruction in Nrf2-deficient mice. *Sci Rep*. 2017;**7**:8801. Doi: 10.1038/s41598-017-08054-2.

32. Kanda Y. Investigation of the freely available easy-to-use software ‘EZR’ for medical statistics. *Bone Marrow Transplant*. 2013;**48**:452-458.

33. Formigli L, Lombardo LD, Adembri C, Brunelleschi S, Ferrari E, Novelli GP. Neutrophils as mediators of human skeletal muscle ischemia-reperfusion syndrome. *Hun Pathol*. 1992;**23**:627-634.

34. Bolisetty S, Agarwal A. Neutrophils in acute kidney injury: not neutral any more. *Kidney Int*. 2009;**75**:674-676.

35. Finsterbusch M, Kitching AR, Hickey MJ. Imaging Leukocyte Responses in the Kidney. *Transplantation*. 2017;**101**:506-516.

36. Kashiwagi E, Tonomura Y, Kondo C, et al. Involvement of neutrophil gelatinase-associated lipocalin and osteopontin in renal tubular regeneration and interstitial fibrosis after cisplatin-induced renal failure. *Exp Toxicol Pathol*. 2014;**66**:301-311.

37. Dong X, Bachman LA, Miller MN, Nath KA, Griffin MD. Dendritic cells facilitate accumulation of IL-17 T cells in the kidney following acute renal obstruction. *Kidney Int*. 2008;**74**:1294-1309.

38. Kelly KJ1, Williams WW Jr, Colvin RB, et al. Intercellular adhesion molecule-1-deficient mice are protected against ischemic renal injury. *J Clin Invest*.

1996;**97**:1056-1063.

39. Rossaint J, Zarbock A. Tissue-specific neutrophil recruitment into the lung, liver, and kidney. *J Innate Immun*. 2013;**5**:348-357.

40. Block H, Herter JM, Rossaint J, et al. Crucial role of SLP-76 and ADAP for neutrophil recruitment in mouse kidney ischemia-reperfusion injury. *J Exp Med*. 2012;**209**:407-421.

41. Benli E, Ayyildiz SN, Cirrik S, et al. The effect of tadalafil therapy on kidney damage caused by sepsis in a polymicrobial septic model induced in rats: a biochemical and histopathological study. *Int Braz J Urol*. 2017;**43**:345-355.

42. Lledo-Garcia E, Rodriguez-Martinez D, Cabello-Benavente R, et al. Sildenafil improves immediate posttransplant parameters in warm-ischemic kidney transplants: experimental study. *Transplant Proc*. 2007;**39**:1354-1356.

43. Porst H, Padma-Nathan H, Giuliano F, Anglin G, Varanese L, Rosen R. Efficacy of tadalafil for the treatment of erectile dysfunction at 24 and 36 hours after dosing: a randomized controlled trial. *Urology*. 2003;**62**:121-125.

44. Patel NN, Lin H, Toth T, et al. Phosphodiesterase-5 inhibition prevents postcardiopulmonary bypass acute kidney injury in swine. *Ann Thorac Surg*. 2011;**92**:2168-2176.

45. Bektas S, Karakaya K, Can M, et al. The effects of tadalafil and pentoxifylline on apoptosis and nitric oxide synthase in liver ischemia/reperfusion injury. *Kaohsiung J Med Sc*i. 2016;**32**:339-347.

46. Zhao L, Mason NA, Morrell NW, et al. Sildenafil inhibits hypoxia-induced pulmonary hypertension. *Circulation*. **2001**;104:424-428.

47. Elrod JW, Greer JM, Lefer DJ. Sildenafil-mediated acute cardioprotection is independent of the NO/cGMP pathway. *Am J Physiol Heart Circ Physiol*. **2007**;292:H342-347.

48. Guzeloglu M, Yalcinkaya F, Atmaca S, et al. The beneficial effects of tadalafil on renal ischemia-reperfusion injury in rats. *Urol Int*. 2011;**86**:197-203.

49. Sogawa Y, Nagasu H, Itano S, et al. The eNOS-NO pathway attenuates kidney dysfunction via suppression of inflammasome activation in aldosterone-induced renal injury model mice. *PLoS One*. 2018;**13**: e0203823. doi: 10.1371/journal.pone.0203823. eCollection 2018.

50. Nagasu H, Satoh M, Kidokoro K, et al. Endothelial dysfunction promotes the transition from compensatory renal hypertrophy to kidney injury after unilateral nephrectomy in mice. *Am J Physiol Renal Physiol*. 2012;**302**:F1402-1408.

51. Kucuk A, Yucel M, Erkasap N, et al. The effects of PDE5 inhibitory drugs on renal ischemia/reperfusion injury in rats. *Mol Biol Rep*. **2012**;39:9775-9782.

52. McAuley IW, Kim NN, Min K, et al. Intracavernosal sildenafil facilitates penile erection independent of the nitric oxide pathway. *J Androl*. **2001**;22:623-628.

53. Medina P, Segarra G, Vila JM, et al. Effects of sildenafil on human penile blood vessels. *Urology*. **2000**;56:539-543.

54. Qin P, Tang X, Elloso MM, Harnish DC. Bile acids induce adhesion molecule expression in endothelial cells through activation of reactive oxygen species, NF-kappaB, and p38. *Am J Physiol Heart Circ Physiol*. 2006;**291**:H741-717.

55. Kidokoro K, Satoh M, Nagasu H, et al. Tacrolimus induces glomerular injury via endothelial dysfunction caused by reactive oxygen species and inflammatory change. *Kidney Blood Press Res*. 2012;**35**:549-557.

56. Awad AS, Rouse M, Huang L, et al. Compartmentalization of neutrophils in the kidney and lung following acute ischemic kidney injury. *Kidney Int*. 2009;**75**:689-698.

57. Cardini B, Fodor M, Hermann M, et al. Live Confocal Imaging as a Novel Tool to Assess Liver Quality: Insights From a Murine Model. *Transplantation*. 2020;**104**:2528-2537.

FIGURE LEGENDS

Figure 1. Neutrophil infiltration during reperfusion in real-time imaging..

A representative serial images showing neutrophil infiltration into the glomerulus after ischemia/reperfusion over time. G: glomerulus. T: tubule. PTC: peritubular capillary. AA: afferent arteriole. Red; BSA-Alexa594, Green; Ly-6G-Alexa488. Bar = 50μm. a) Neutrophil infiltration into the glomerulus increased with time after reperfusion. b) The neutrophil influx into the glomerulus from afferent arteriole. The images were taken every 4 min starting 15 min after reperfusion. c) The neutrophils present in the tubular lumen 3 hours after reperfusion. d) The neutrophils present inside and outside of peritubular capillaries 24 hours after reperfusion.

Figure 2. Periodic acid-Schiff staining and evaluation of tubular injury 24 h after renal IRI.

Tadalafil decreased the tubulointerstitial injury in both WT and eNOS-KO mice after renal IRI. a) Periodic acid-Schiff staining was performed to evaluate tubular injury 24 h after renal IRI (n=3/control groups; n=6/IRI and IRI + PDE5 groups).

Bar = 100μm. d) Tubular injury score 24 h after renal IRI (0-4). *, P<0.05. Data are shown as mean ± SEM.

Figure 3. FACS analysis of neutrophils in whole kidney tissues 3 h after reperfusion injury.

Tadalafil prevented the accumulation of neutrophils in whole kidney tissues from WT mice and eNOS-KO mice (n=3/control group; n=6/IRI and IRI + PDE5 groups). a) Representable flow cytometric plots of neutrophils from whole kidney tissues. b) Fractions of CD11b+ and Ly6G+ cells among total CD45+ live cells. *, P<0.05.

Figure 4. Multiphoton real-time imaging which focused on neutrophil infiltration into the glomerulus.

The administration of tadalafil reduced neutrophil influx into the glomerulus in both WT and eNOS-KO groups. G: glomerulus, US: urinary space, T: tubules. Red; BSA-Alexa594, Green; Ly-6G-Alexa488. Bar = 50μm. a) A representative series of images showing neutrophil infiltration into the glomerulus over time. b)

Percentage of neutrophil positive area in the glomerulus 1 h after renal IRI. *, P<0.05. Data are shown as mean ± SEM.

Figure 5. ICAM-1 and VCAM-1 mRNA levels in kidneys after reperfusion injury.

Expression of ICAM-1 and VCAM-1 mRNAs in each group 3 h after renal ischemia/reperfusion injury. The mRNA levels of ICAM-1 (a) and VCAM-1 (b) were quantitatively analyzed by real-time PCR. *, P < 0.05. Data are shown as mean ± SEM.

Figure 6. KIM-1 staining 24 h after renal IRI.

Tubular cell injury was decreased by tadalafil in a mouse renal ischemia/reperfusion model. a) KIM-1 staining showing renal cells damaged by inflammation 24 h after renal IRI. Bar = 100μm. b) KIM-1-positive area (%). Data are shown as mean ± SEM. *, P < 0.05.

Supplemental video 1. Representative video of collapsed glomerulus bulging immediately after reperfusion with reinitiation of blood flow. The diameter of the glomerulus expanded from 40μm to 60μm. G: glomerulus. Red; BSA-Alexa594, Green; Ly-6G-Alexa488.

Supplemental video 2. Supplemental video 2. Representative video showing influx of neutrophils into the glomerulus from afferent arterioles. G: glomerulus. AA: afferent arteriole. Red; BSA-Alexa594, Green; Ly-6G-Alexa488. Time per frame: 4 min.

Supplemental video 3. Representative video of neutrophils rolling in the peritubular capillary plexus 24 h after renal ischemia/reperfusion. Bar, 100μm. Thirty min of continuous shooting was compressed. The most visible neutrophil moved about 3μm/min.