博士論文

論文題目　Inhibition of VAP-1 enzyme activity attenuates renal ischemia–reperfusion injury via suppression of neutrophil infiltration

（VAP-1 酵素活性の阻害は、好中球浸潤の抑制を介して腎虚血再灌流障害を軽減する）

氏　　名　　田中　真司

ABSTRACT

Vascular adhesion protein-1 (VAP-1) is an adhesion molecule expressed in endothelial cells. This molecule is unique because it also acts as an ectoenzyme, catalyzing oxidative deamination of primary amines. While inhibition of VAP-1 enzyme activity ameliorated several inflammatory disease models in various organs, no report has been published on the effect of VAP-1 inhibition in acute kidney injury (AKI) models. Thus, I examined the effect of a specific VAP-1 inhibitor in a rat model of renal ischemia–reperfusion (IR) injury. Immunofluorescence and immunoblotting analysis suggested that VAP-1 is expressed predominantly in pericytes of rodent kidneys. A specific VAP-1 inhibitor significantly reduced renal/plasma VAP-1 enzyme activity 48 h after surgery. VAP-1 inhibition significantly ameliorated renal IR injury 48 h after surgery (as determined by BUN and plasma creatinine levels, histological tubular injury, and KIM-1 mRNA expression in the whole kidney). The improvement of renal injury was significantly associated with a decreased number of neutrophils, but not macrophages, infiltrating into the corticomedullary junction. Notably, none of other adhesion molecules (VCAM-1, ICAM-1, E-selectin, and P-selectin) exhibited altered expression levels with VAP-1 inhibition. In conclusion, the present study suggests that VAP-1, which is expressed in pericytes of the kidney, controls neutrophil infiltration in renal IR

injury, potentially offering a promising view that VAP-1 inhibition might be used as a novel therapeutic strategy in ischemic AKI.

INTRODUCTION

Acute kidney injury (AKI) has attracted attention as a primary clinical concern; AKI has a much greater impact on clinical outcomes than previously believed. For example, AKI occurring after cardiac surgery has been strongly associated with elevated mortality [1], with greater AKI severity directly associated with higher mortality rates [2]. Additionally, some AKI episodes can result in transition to chronic kidney disease (CKD) [3, 4]. Thus, there is an urgent need to develop a novel therapeutic strategy against AKI.

Although the etiology of AKI is variable, ischemia and sepsis constitute the clinically most important causes, which account for two thirds of all cases [5]. In addition, ischemia is regarded as an important player in septic AKI considering the correlation between episodes of hypotension and the incidence of AKI. Based on these, the renal ischemia–reperfusion (IR) injury model has frequently been used in studies of AKI [6].

In renal IR injury, inflammation caused by infiltration of leukocytes, especially neutrophils, into the kidney plays a critical role in pathogenesis [7]. Leukocyte infiltration initiates with rolling and adhesion of leukocytes on activated endothelial cells. Expression levels of adhesion molecules, such as intercellular adhesion molecule

(ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and selectins on endothelial cells are upregulated in ischemia, and blocking these molecules were shown to successfully ameliorate renal IR injury in a number of studies [8-12]. However, no drug has been approved for the treatment of AKI in humans. For example, an anti-ICAM-1 antibody in recipients of renal transplants failed to demonstrate decreased rates of delayed graft function or acute rejection in a randomized controlled trial [13]. Thus, it is valuable to seek other strategies to block leukocyte infiltration into injured kidneys.

Vascular adhesion protein-1 (VAP-1) is another adhesion molecule expressed in endothelial cells [14, 15]. This molecule is unique because it also acts as an ectoenzyme, catalyzing oxidative deamination of primary amines ($R\text{-}CH_2\text{-}NH_2 + O_2 + H_2O \rightarrow R\text{-}CHO + NH_3 + H_2O_2$). Although several primary amines such as benzylamine are known as efficient substrates, the entire spectrum of substrates in vivo remains incompletely understood.

VAP-1 is a homodimeric transmembrane glycoprotein with a molecular weight of 170–180 kDa [16, 17]. In addition to the transmembrane form, a soluble form of VAP-1 (sVAP-1) is detected in circulation. sVAP-1 is thought to be produced through shedding of the transmembrane form; however, the function and the regulation of this shedding is yet to be determined. In various organs, VAP-1 expression has been

confirmed in smooth muscle cells, pericytes, and adipocytes, as well as in endothelial cells [18-21]. However, little is known about the functional roles of VAP-1 attached to non-endothelial cells.

To date, VAP-1 upregulation enhances leukocyte infiltration in inflammatory tissues, and its enzymatic activity has been reported to play a pivotal role in some studies [22]. While inhibition of VAP-1 enzyme activity ameliorated several inflammatory disease models in various organs such as the intestine [23, 24], liver [25], lung [23, 26, 27], eye [28], and brain [29-32], no study has addressed the effect of VAP-1 inhibition in AKI models.

Based on the background discussed above, I hypothesized that inhibition of VAP-1 enzyme activity would ameliorate renal IR injury, via suppression of leukocyte infiltration. To address this hypothesis, I examined the effect of a specific VAP-1 inhibitor in a rat model of renal IR injury.

METHODS

Animal Models

Eight-week-old male Sprague–Dawley rats (Nippon Seibutsu Zairyo Center, Tokyo, Japan) were housed in an air-conditioned room under a 12-h light/dark cycle with free access to food and water. Rats were randomly divided into vehicle and drug (VAP-1 inhibitor, RTU-1096) groups. A specific VAP-1 inhibitor, RTU-1096, was generously provided by R-Tech Ueno (Tokyo, Japan). RTU-1096 has a high specificity for VAP-1 against other monoamine oxidases (MAOs); IC_{50} for rat VAP-1, human VAP-1, human MAO-A, human MAO-B are 0.4 nM, 0.9 nM, >100 μM, >100 μM, respectively (data provided by R-Tech Ueno). In the initial pilot study, RTU-1096 was administered by oral gavage (50 mg/kg/day) at -12, 0, 12, 24, 36 h. However, this protocol failed to show protective effects against renal IR injury (data not shown). Thus, RTU-1096 was mixed in feed (0.05%) considering the possibility that VAP-1 enzyme activity could recover during the gavage interval. These doses were decided based on pharmacokinetic data of this inhibitor in rats (data not shown). The actual dose of RTU-1096 was about 40 mg/kg/day (intake, 24 g/day; body weight, 300 g). RTU-1096 or vehicle was administered to rats from 7 days before IR surgery until sacrifice. Rats were subjected to left renal ischemia for 45 min following right nephrectomy and sacrificed at 6 h (n =

5 each) or 48 h (n = 10 each) after IR operation. In the sham groups (n = 3 each), rats underwent similar procedures except for left renal ischemia.

All animal experiments were approved by the ethical committee of the Graduate School of Medicine, the University of Tokyo (P15-073), and performed in accordance with the guidelines established by the Committee on Ethical Animal Care and Use at the University of Tokyo.

Determination of VAP-1 Enzyme Activity

For radioenzymatic determination of VAP-1 enzyme activity, the original procedure described by Yu PH *et al* [33] was modified. In brief, samples (plasma or kidney homogenates) and reaction buffer including a monoamine oxidase inhibitor were pre-incubated at room temperature for 20 min. Following this, samples were incubated in the presence of ^{14}C-labeled benzylamine at 37°C for 2 h. Enzyme reactions were terminated with addition of 2M citric acid. The oxidized products were extracted into toluene/ethyl acetate (1:1 vol/vol). Radioactivity of ^{14}C-labeled benzaldehyde, a metabolite of the ^{14}C-labeled benzylamine, was assessed using a liquid scintillation counter (LSC-6100, Aloka, Tokyo, Japan). Total protein was calculated with the Bradford method (Quick Start™ Bradford Protein Assay, Bio-rad, Hercules, CA, USA), using a microplate reader (Varioskan, Thermo Electron, Vantaa, Finland).

Biochemical Measurement

Concentrations of blood urea nitrogen (BUN) and plasma creatinine (Cr) were measured with commercial kits (Wako, Osaka, Japan).

Histological Evaluation

Formalin-fixed, paraffin-embedded tissue was sectioned (3 μm thick), dewaxed with Histoclear® (National Diagenostics, Atlanta, GA, USA), and rehydrated through graded ethanols. Periodic acid-Schiff (PAS) staining was used in semiquantitative evaluation of tubular injury. Tubular injury scores were graded (0–4) according to the following criteria [34]:

0. No change.
1. Mitosis and necrosis of individual cells.
2. Necrosis of all cells in adjacent proximal convoluted tubules, with survival of surrounding tubules.
3. Necrosis confined to the distal third of the proximal convoluted tubule, with a band of necrosis extending across the inner cortex.
4. Necrosis affecting all three segments of the proximal convoluted tubule.

Randomly selected 10 fields (×200) were scored and the highest score was adopted. All evaluations were performed in a blinded manner.

Immunohistochemistry

For detection of neutrophils, naphthol AS-D chloroacetate (Specific Esterase) kit (Sigma-Aldrich, St. Louis, MO, USA) was used according to manufacturer's recommendations. For detection of macrophages, indirect immunoperoxidase staining was performed on methyl Carnoy's-fixed, paraffin-embedded tissue sections (3 μm thick). After dewaxing and rehydration, blocking of pseudoperoxidase with 3% hydrogen peroxide and nonspecific protein binding was performed. Following incubation overnight at 4°C with mouse monoclonal anti-rat macrophage antibody (ED-1, Chemicon, Temecula, CA, USA), sections were incubated for 40 min at room temperature with biotinylated horse anti-mouse IgG antibody (Vector, Burlingame, CA, USA). Then, the sections were incubated with avidin D, horseradish peroxidase (Vector) for 30 min at room temperature. Color was developed via incubation with diaminobenzidine (DAB, Wako) and hydrogen peroxide. Neutrophil and macrophage numbers were counted in 10 randomly selected fields (×400) of corticomedullary junction in a blinded manner.

Immunofluorescence Study

Periodate-Lysine-Paraformaldehyde-fixed kidneys were embedded in O.C.T. compound (Sakura Finetek Japan, Tokyo, Japan) and frozen on dry ice. The block was cut into 4

μm sections and incubated overnight at 4°C with mixed primary antibodies (rabbit polyclonal anti-VAP-1 antibody (Abcam, Tokyo, Japan) and mouse monoclonal anti-rat RECA-1 antibody (AbD Serotec, Oxford, UK)/mouse monoclonal anti-PDGFRβ antibody (Novus, Littleton, CO, USA)). Subsequently, sections were incubated with a mixture of FITC-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL, USA) and TRITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) for 60 min at room temperature in dark conditions. A TCS-SP5 confocal fluorescence microscope (Leica Microsystems, Tokyo, Japan) was used for evaluation.

Total RNA Isolation and Real-time Quantitative PCR

Mechanically homogenized whole-kidney samples were used in RNA quantification. Total RNA was isolated with RNAiso® (TAKARA BIO, Shiga, Japan), and reverse-transcribed with RT Master Mix® (TAKARA BIO). Following this, cDNA was subjected to real-time quantitative PCR using THUNDERBIRD qPCR Mix® (Toyobo, Tokyo, Japan) and a CFX96 Real Time System® (Bio-Rad), according to manufacturer's protocols. Primer sequences are listed in Table 1.

Table 1: A list of primers used in this study.

Gene	Species	F/R	Sequence (5'→3')
Vcam-1	Rat	Forward	TTGTTCAAGAGAAACCATTTACTGT
		Reverse	GCTCATCCTCAACACCCACA
Icam-1	Rat	Forward	CGGTGCTCAGGTATCCATCC
		Reverse	CTCGCTCTGGGAACGAATACA
Kim-1	Rat	Forward	ATGGCTGCCCTCAGTTTTCTC
		Reverse	AATCTACAGAGCCTGGAAGAAGC
E-selectin	Rat	Forward	TGCAGGGGTACAGTGTTCAA
		Reverse	GGCAGCTACTAGCAGGAACG
P-selectin	Rat	Forward	GACGGGTCAAGAGAGGACA
		Reverse	TCGATTTACAAGCTCAGAGATCA
Cxcl1	Rat	Forward	ATGCTAAAGGGTGTCCCCAA
		Reverse	TTGTCAGAAGCCAGCGTTCA
Cxcl2	Rat	Forward	AACCATCAGGGTACAGGGGT
		Reverse	CAACCCTTGGTAGGGTCGTC
Mcp-1	Rat	Forward	GACAGAGGCCAGCCCAGAAACC
		Reverse	CAACAGGCCCAGAAGCGTGACA

β-actin	Rat	Forward	CTTTCTACAATGAGCTGCGTG
		Reverse	TCATGAGGTAGTCTGTCAGG
Aoc3 (VAP-1)	Mouse	Forward	TTGTGGGGACGGTGCTTGTG
		Reverse	GTTCTTCAGCCCTGCCACATC

Cell Culture

Isolated mouse kidney pericytes were gifted from Dr. Jeremy Duffield (University of Washington). Cells were cultured in DMEM/F12 (Sigma-Aldrich) with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 1% penicillin/streptomycin, and 1% insulin-transferrin-selenium (Invitrogen, Carlsbad, CA, USA), in plates precoated with 0.2% gelatin.

Immunoblotting

Cell pellets of cultured mouse kidney pericytes were resuspended in RIPA buffer (50 mmol/l Tris-HCl (pH8.0), 150 mmol/l sodium chloride, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1.0% Triton X-100) with a proteinase inhibitor cocktail (cOmplete Mini®, Nippon-Roche, Tokyo, Japan), and denatured with incubation in sample buffer (2% sodium dodecyl sulfate, 10% glycerol, 60 mM Tris (pH 6.8), 10 mM dithiothreitol, and 0.01% bromophenol blue) for 5 min at 96°C. Proteins were separated with 10% SDS polyacrylamide gel electrophoresis and transferred onto a

polyvinylidene fluoride membrane. Nonspecific protein binding was blocked with 5% skim milk in Tris-buffered saline (pH 7.4), containing 0.5% Tween 20. Membranes were incubated with the primary antibody (rabbit polyclonal anti-VAP-1 antibody (Abcam)) at 4°C overnight, and then incubated at room temperature for 45 min with HRP-conjugated goat anti rabbit IgG antibody (Bio-Rad). Bands were observed with an enhanced chemiluminescence system (ECL Plus® and Image Quant LAS 4000®, GE Healthcare, Tokyo, Japan).

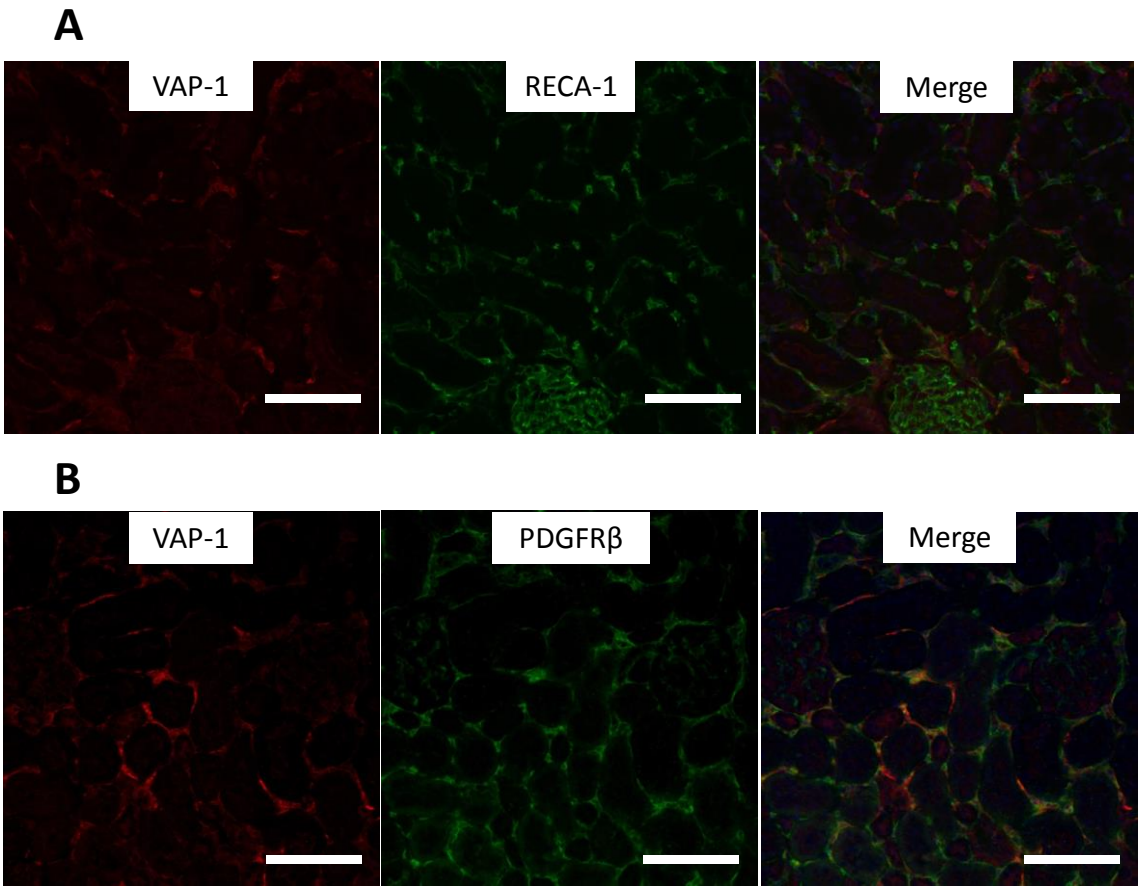
Statistical Analysis

All values are expressed as mean ± SEM. Data for two groups were analyzed using Mann–Whitney U test in semiquantitative evaluation of tubular injury, or a Student's two-tailed *t*-test in other data. P-values < 0.05 were regarded as statistically significant. All analyses were performed with JMP® 11 software (SAS Institute Inc., Cary, NC, USA).

RESULTS

VAP-1 Expression of the Kidney Pericytes

Immunofluorescence analysis using sham-operated rat kidneys suggested that VAP-1 is expressed predominantly in pericytes rather than in endothelial cells (Figure 1A, 1B). In addition, the existence of VAP-1 mRNA and protein was confirmed in cultured pericytes isolated from mouse kidneys (Figure 1C). Although I tried to confirm the localization of VAP-1 also in IR-injured kidneys with immunofluorescence analysis, it was difficult to obtain clear images due to nonspecific signals from injured tubules and casts.



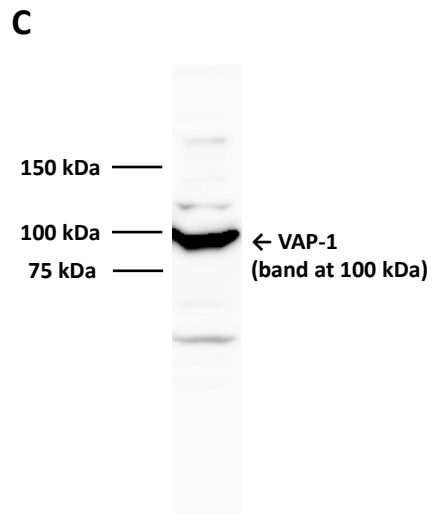


Figure 1. Predominant VAP-1 expression in pericytes of the kidney.
(A, B) Immunofluorescence analysis for detection of VAP-1, RECA-1 (endothelial marker), and PDGFRβ (pericyte marker) in the sham-treated rat kidney. Staining for VAP-1, RECA-1, PDGFRβ, and their corresponding merged images are shown. Scale bar, 100 μm. (C) Immunoblotting of VAP-1 in whole-cell extract of cultured mouse kidney pericytes.

Renal and Plasma VAP-1 Enzyme Activity following IR Surgery and the Effect of VAP-1 Inhibitor

First, renal/plasma VAP-1 enzyme activity was examined at 0 h (sham), 6 h, and 48 h after surgery, to determine whether enzymatic activity had been altered. IR surgery significantly increased plasma VAP-1 activity at 6 h, which returned to the baseline at 48 h. In contrast, renal VAP-1 activity did not significantly change at time points I studied (Figure 2). A specific inhibitor, RTU-1096, significantly reduced renal/plasma VAP-1 enzyme activity at each time point.

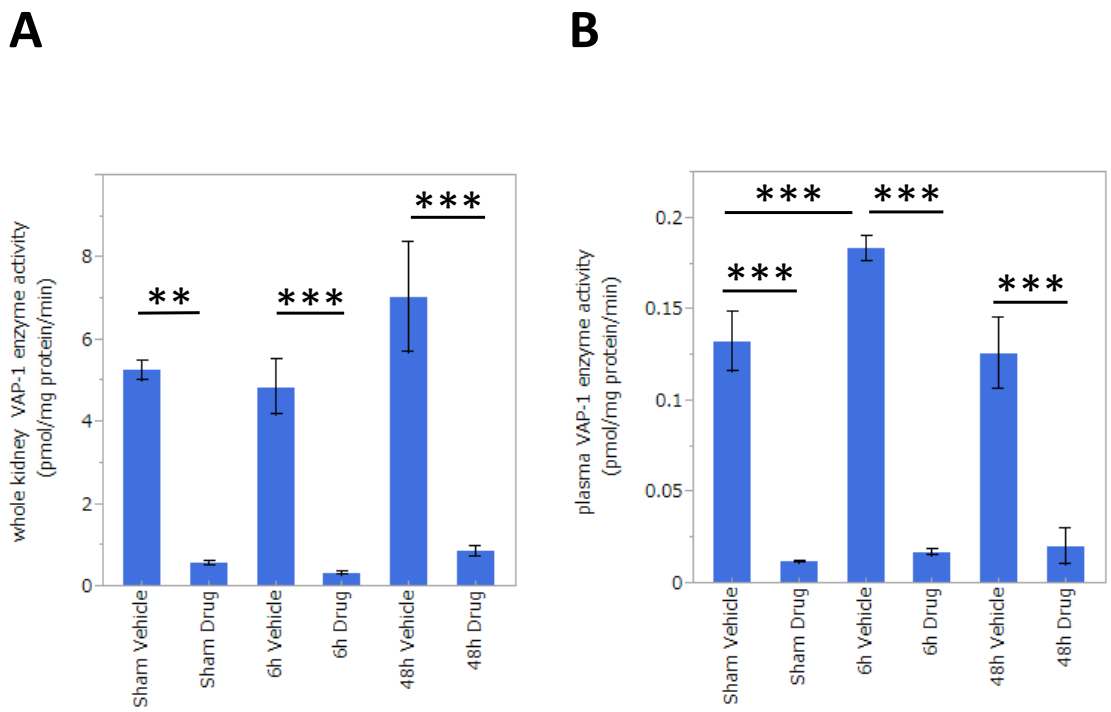


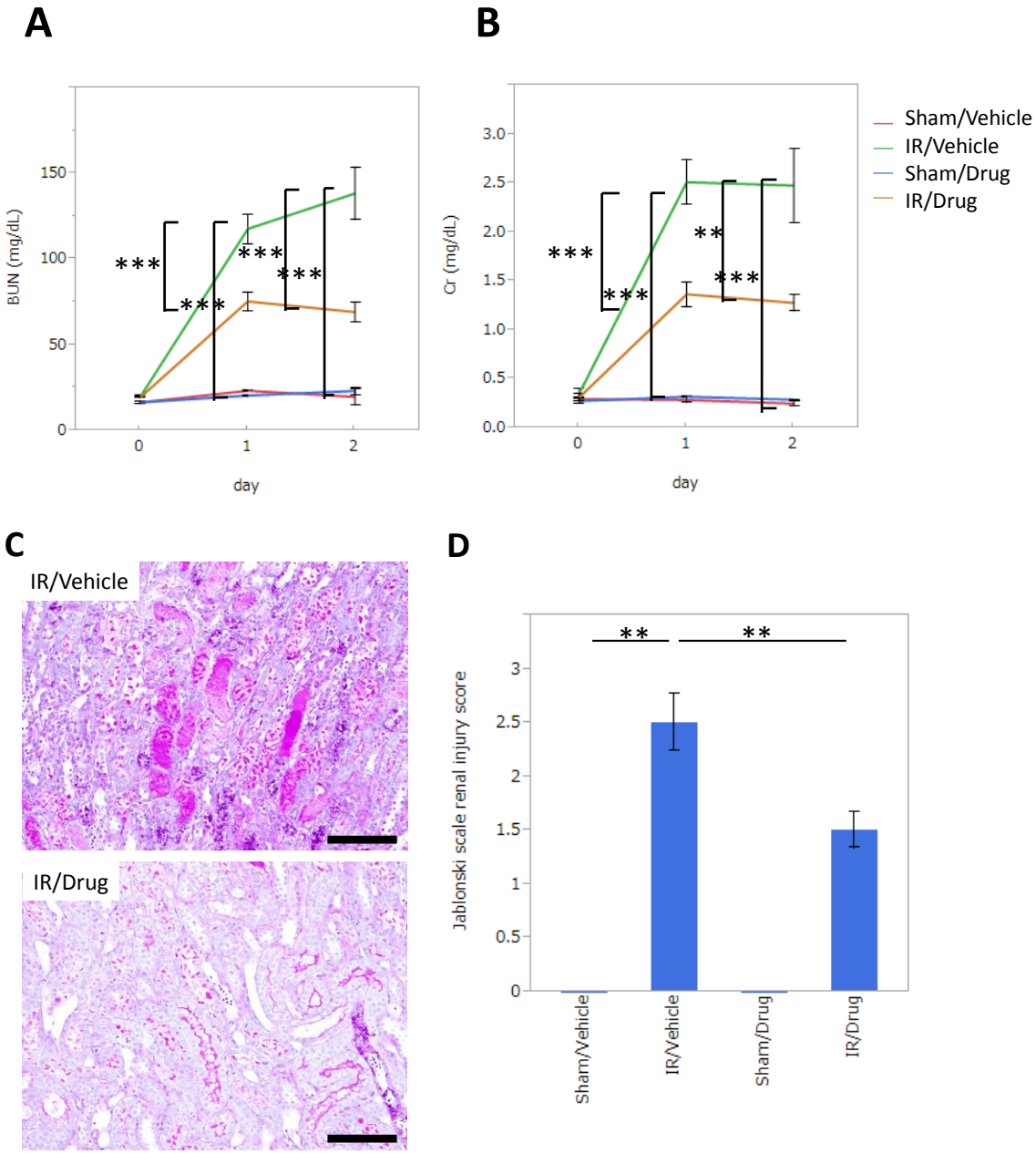
Figure 2. Comparison of renal/plasma VAP-1 enzyme activity at 0 h (sham), 6 h, and 48 h after surgery among test groups.

(A, B) Whole kidney (A) or plasma (B) VAP-1 enzyme activity in experimental rats at 0 h (sham), 6 h, and 48 h after surgery. (Sham Vehicle, n = 3; Sham Drug, n = 3; 6h Vehicle, n = 5; 6h Drug, n = 5; 48h Vehicle, n = 10; 48h Drug, n = 10; ** $p < 0.01$, *** $p < 0.001$)

Amelioration of Renal IR Injury by VAP-1 Inhibition

In the vehicle groups, IR surgery resulted in marked elevations of BUN and plasma Cr levels both at day 1 and day 2, suggesting that IR injury in this study was robust (Figure 3A, 3B). In IR groups, VAP-1 inhibition was associated with significantly lowered BUN and plasma Cr levels both at day 1 and day 2. Semiquantitative analysis of histological

tubular injury also demonstrated that the animals in the IR/Vehicle group exhibited significant degrees of tubular injury, which became less prominent in the IR/Drug group at 48 h (Figure 3C, 3D). In accordance with these biochemical and histological observations, kidney injury molecule (KIM)-1 (a representative tubular injury marker [35, 36]) was also significantly downregulated with VAP-1 inhibition (Figure 3E).



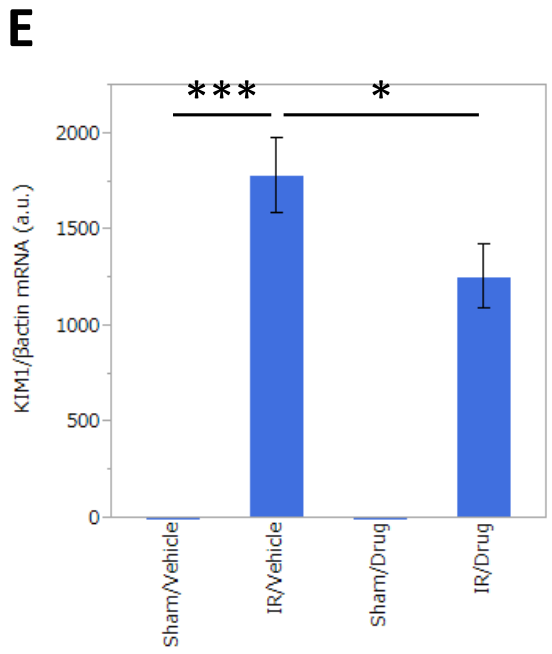


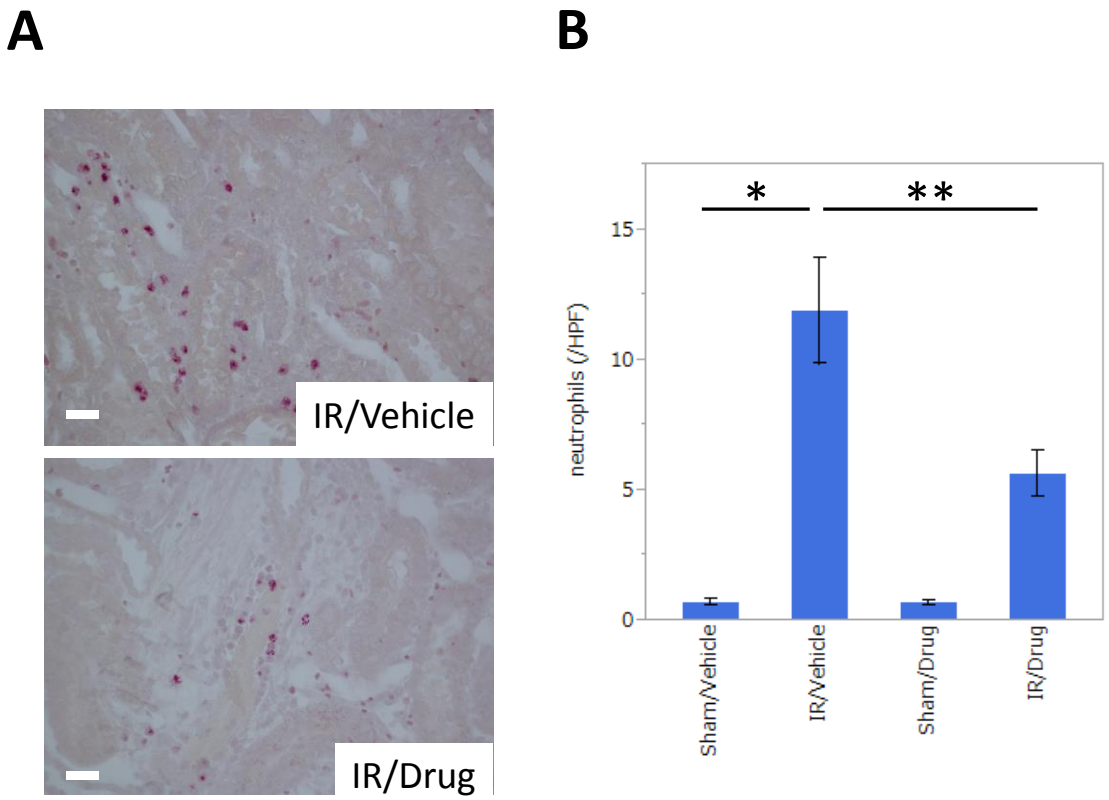
Figure 3. VAP-1 inhibition ameliorated renal IR injury.

(A-E) Rats were subjected to left renal ischemia for 45 min following right nephrectomy and sacrificed 48 h after IR operation. In the sham groups, rats underwent similar procedures except for left renal ischemia. (A, B) Time course of BUN (A) and plasma Cr (B). (C) Histological analysis with PAS staining. Scale bars, 100 μm. (D) Semiquantitative analysis of histological tubular injury. (E) Quantitative PCR of KIM-1 mRNA in the whole kidney. (Sham/Vehicle, n = 3; IR/Vehicle, n = 10; Sham/Drug, n = 3; IR/Drug, n = 10; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

VAP-1 Inhibition was Associated with Suppressed Neutrophil Infiltration at 48 h after Renal IR

VAP-1 has been reported to play an important role in leukocyte trafficking in inflammatory tissue. Therefore, I investigated whether neutrophil/macrophage infiltration into IR-injured kidneys was influenced by VAP-1 inhibition, because these cells are critical contributors in the pathogenesis of renal IR injury. The number of

neutrophils infiltrating into the corticomedullary junction, where leukocyte infiltration was most pronounced, was significantly increased at 48 h after IR injury, which was suppressed by VAP-1 inhibition (Figure 4A, 4B). The number of macrophages infiltrating into the corticomedullary junction was also significantly increased at 48 h after IR injury. In contrast, however, the number of macrophages did not differ between the IR/Vehicle and IR/Drug groups (Figure 4C, 4D).



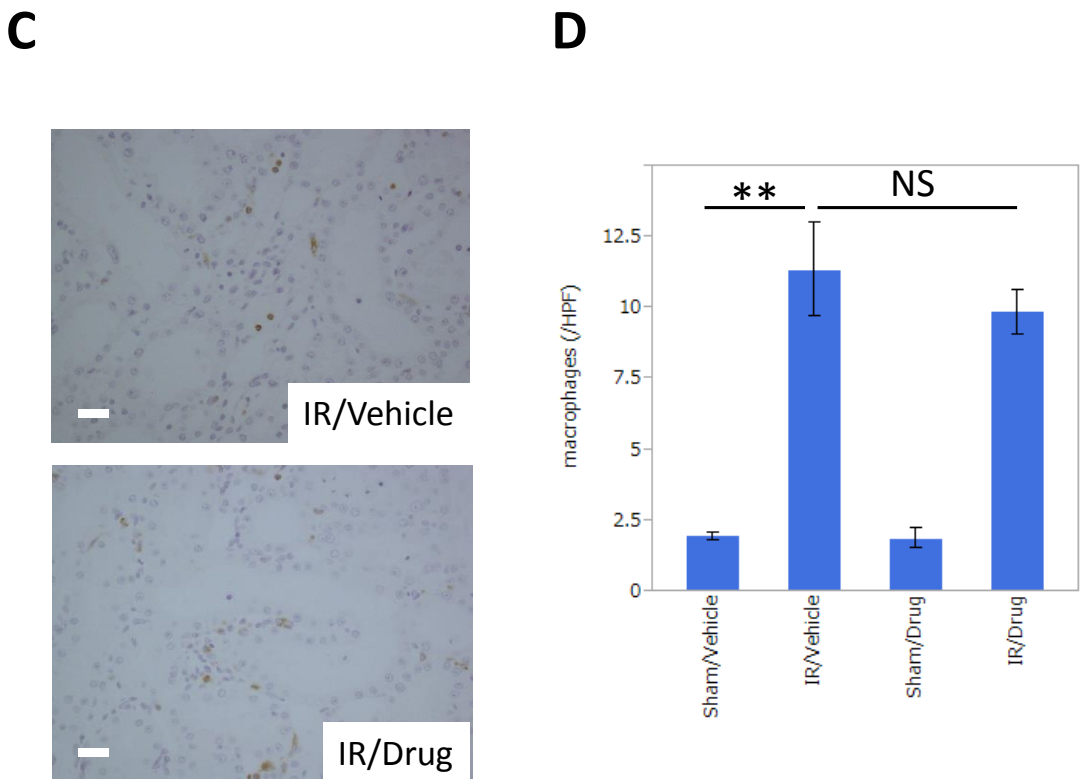


Figure 4. VAP-1 inhibition suppressed neutrophil, but not macrophage, infiltration into the IR-injured kidney at 48 h.

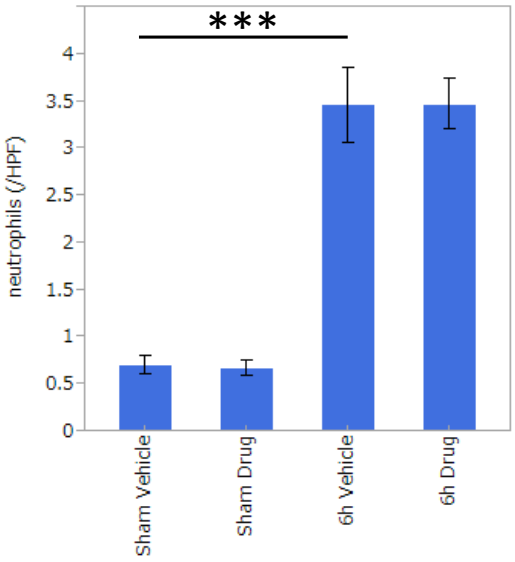
(A, B) Infiltrating neutrophils (stained by naphthol AS-D chloroacetate) in the corticomedullary junction at 48 h after IR injury. (C, D) Infiltrating macrophages (ED-1 positive cells) in the corticomedullary junction at 48 h after IR injury. Scale bars, 30 μm. (Sham/Vehicle, n = 3; IR/Vehicle, n = 10; Sham/Drug, n = 3; IR/Drug, n = 10; * $p < 0.05$, ** $p < 0.01$)

Changes in Neutrophil Chemokines and Other Adhesion Molecules by VAP-1 Inhibition

To gain insight into the mechanism of decreased neutrophil infiltration with VAP-1 inhibition, I investigated the time course of neutrophil infiltration, chemokines, and

other adhesion molecules. The number of infiltrating neutrophils was not reduced by VAP-1 inhibition at 6 h after IR (Figure 5A). Consistently, whole-kidney expression levels of CXCL-1 and CXCL-2, key neutrophil chemokines significantly upregulated in renal IR injury, were not altered by VAP-1 inhibition at 6 h, but again remarkably suppressed at 48 h (Figure 5B, upper panels). In contrast, the expression level of MCP-1, a representative macrophage chemokine, was not affected by VAP-1 inhibition either at 6 h or at 48 h (Figure 5B, lower panel). These results are in accordance with the observed changes in numbers of infiltrating neutrophils/macrophages.

A



B

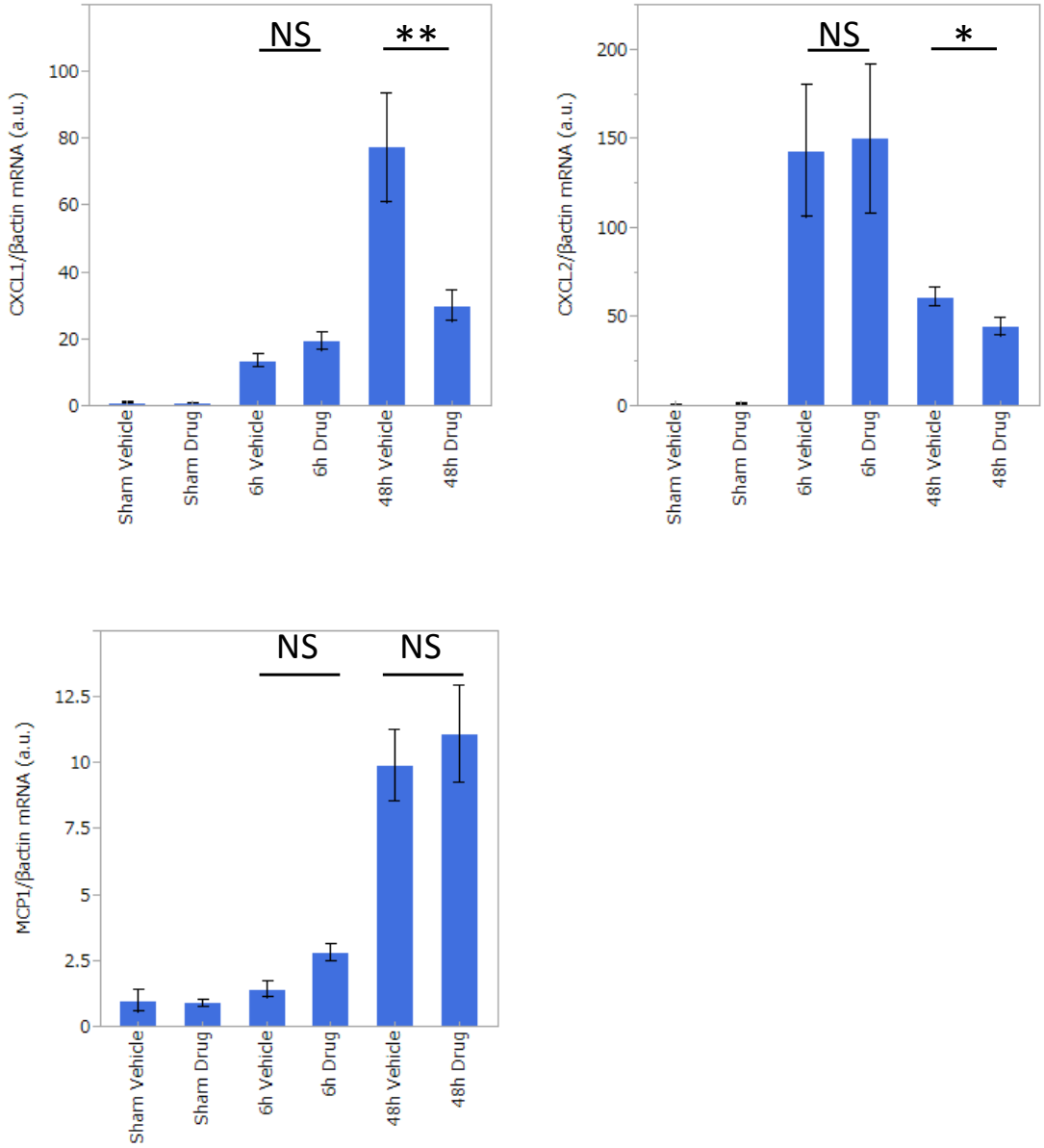
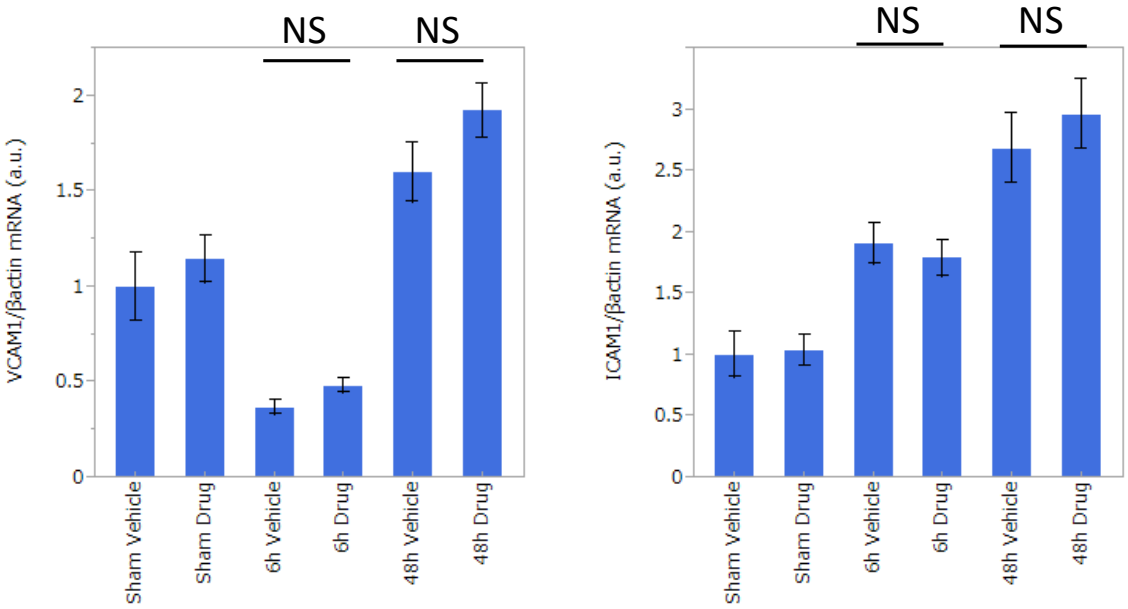


Figure 5. The time-course changes of neutrophil infiltration and its associated chemokines in IR-injured kidneys.

(A) The number of infiltrating neutrophils at 6 h after IR injury. (B) Quantitative PCR of CXCL-1 and CXCL-2 (neutrophil chemokines) and MCP-1 (a macrophage chemokine) in the whole kidneys at 0 h (sham), 6 h, and 48 h after IR. (Sham Vehicle, n = 3; Sham Drug, n = 3; 6h Vehicle, n = 5; 6h Drug, n = 5; 48h Vehicle, n = 10; 48h Drug, n = 10; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Finally, I tested the possibility that VAP-1 inhibition also influenced the expression of alternative adhesion molecules which possibly suppress neutrophil infiltration into IR-injured kidneys. To this end, expression levels of VCAM-1, ICAM-1, E-selectin, and P-selectin in the whole kidney (Figure 6) were quantified; however, none of these molecules were affected by VAP-1 inhibition either at 6 h or at 48 h after IR.



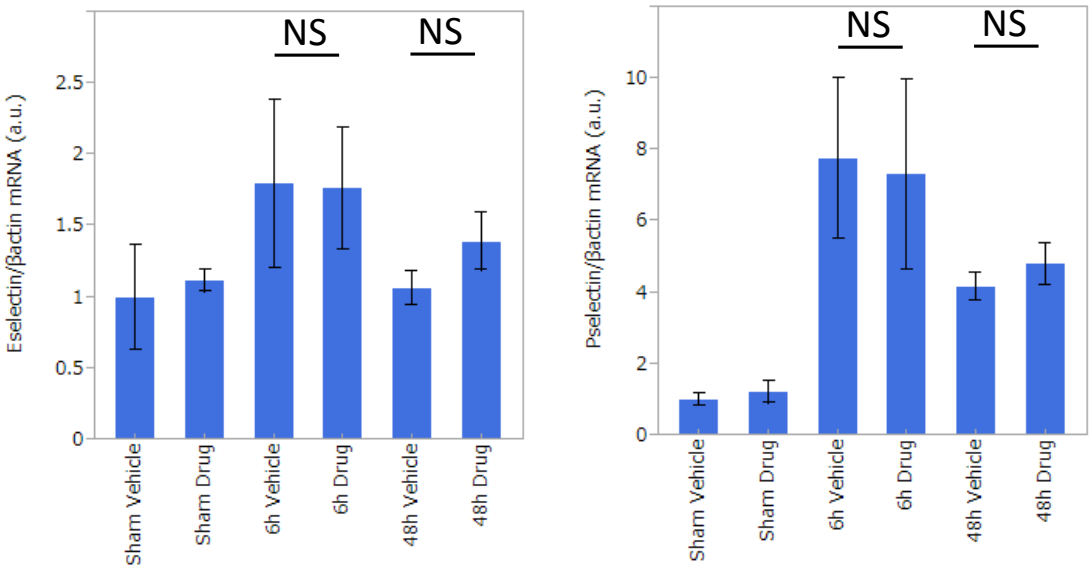


Figure 6. Other adhesion molecules were not affected by VAP-1 inhibition.

Quantitative PCR of VCAM-1, ICAM-1, E-selectin, and P-selectin in the entire kidney at 0 h (sham), 6 h, and 48 h after IR. (Sham Vehicle, n = 3; Sham Drug, n = 3; 6h Vehicle, n = 5; 6h Drug, n = 5; 48h Vehicle, n = 10; 48h Drug, n = 10)

DISCUSSION

In this study, I investigated a pathogenic role of VAP-1 and tested a therapeutic impact of VAP-1 inhibition, using a specific inhibitor, RTU-1096, in a rat model of renal IR injury. VAP-1 is expressed in pericytes of rodent kidneys, and RTU-1096 successfully inhibited VAP-1 enzyme activity in rat kidneys and plasma. While VAP-1 enzyme activity in the kidney was not significantly altered by IR injury, inhibition of its enzyme activity significantly ameliorated renal IR injury. BUN/plasma Cr levels, histological tubular injury, and renal KIM-1 mRNA levels were significantly decreased in the IR/Drug group, as compared with the IR/Vehicle group. Amelioration of kidney injury was associated with decreased neutrophil infiltration into the kidney, and suppressed mRNA levels of neutrophil chemokines (CXCL-1 and CXCL-2) at 48 h after IR surgery. However, macrophage infiltration and MCP-1 expression levels were unchanged with VAP-1 inhibition. Furthermore, VAP-1 inhibition did not alter expression of other major adhesion molecules (VCAM-1, ICAM-1, E-selectin, P-selectin) in the kidney. In summary, VAP-1 inhibition ameliorated renal IR injury, most likely via suppression of kidney neutrophil infiltration.

One novel finding of this study is that VAP-1 is expressed in the pericytes of rodent kidneys. However, the use of one polyclonal antibody available in the market

may hamper interpretation of immunodetection. Further experiments including preincubation of the antibody with epitope peptide and siRNA knockdown of VAP-1 gene in cultured mouse kidney pericytes would raise specificity of findings and further support our view on pericyte expression of VAP-1. To date, many studies on VAP-1 focus on its functional role in endothelial cells, whereas only a few have investigated functions of VAP-1 in cells of non-endothelial lineage, such as pericytes. Recently, Weston et al [25] demonstrated that VAP-1 was expressed in stellate cells of the liver and enhanced recruitment of various types of leukocytes, resulting in inflammation and fibrosis.

Results of the present study suggest that VAP-1 inhibition ameliorates rat renal IR injury via suppression of neutrophil infiltration. In other disease models of distinct organs, VAP-1 has been reported to be involved in the recruitment of lymphocytes and macrophages as well as neutrophils into inflamed tissues [14, 25]. However, it may depend on disease models which type of leukocytes is most affected by VAP-1 inhibition. Indeed, amelioration of inflammation with VAP-1 inhibition has been associated with decreased neutrophil infiltration in various lung injury models [26] and in subarachnoid hemorrhage-associated cerebrovascular dilating dysfunction [29]. One may argue that decreased neutrophil infiltration is secondary to amelioration of injury;

however, milder injury is not necessarily accompanied with decreased leukocyte infiltration in this model [37]. Findings that infiltration of macrophages was not blocked by VAP-1 inhibition, also indicate that VAP-1 inhibition directly suppresses neutrophil infiltration, resulting in amelioration of IR injury. Investigating the effect of VAP-1 inhibition in neutrophil-depleted rats would help to understand more deeply the association between VAP-1 inhibition and suppressed neutrophil infiltration.

Then, by which mechanism does VAP-1 enzyme activity enhance neutrophil recruitment in this model? There are some possibilities. (1) VAP-1 in pericytes may function as an adhesion molecule. Recently, pericytes have emerged as a key player in leukocyte migration in inflamed tissues [38, 39]. For example, in response to inflammatory stimuli, pericytes upregulate ICAM-1 expression, promoting interaction with activated leukocytes [40]. (2) VAP-1 in pericytes may act as a potent neutrophil chemotactic factor. Weston et al [25] demonstrated that stellate cells in the liver secreted enzymatically active VAP-1 (sVAP-1), which exhibited a chemotactic effect to lymphocytes in migration assay. They also showed that this property was dependent, at least in part, on the generation of H_2O_2, an end product of the VAP-1 enzyme reaction. A local gradient of sVAP-1/H_2O_2 may be generated in inflamed tissues, which would, in turn, stimulate neutrophil recruitment. To investigate this possibility, migration assays

using neutrophils and VAP-1 protein with appropriate substrates would be needed. (3) In the present study, expression levels of CXCL-1 and CXCL-2 were significantly lower in the IR/Drug group at 48 h after IR surgery, when compared to those of the IR/Vehicle group. These chemokines are known to be important for neutrophil recruitment in the renal IR injury model [41-43]. Considering that these chemokines are produced in various kidney cells, including pericytes, VAP-1 inhibition may directly inhibit the production of these chemokines, resulting in mitigation of neutrophil infiltration. However, considering that VAP-1 inhibition did not affect CXCL-1 and CXCL-2 levels in the kidney at 6 h after IR, decreased levels of CXCL-1 and CXCL-2 are probably secondary to mitigation in IR injury and neutrophil infiltration. Indeed, an infiltrated neutrophil itself was reported to produce a large amount of IL-17A, thereby inducing CXCL-1 and CXCL-2 expression [44]. (4) Lastly, in the present study, VAP-1 (sVAP-1) enzyme activity in plasma was inhibited by RTU-1096. This might lead to suppression of neutrophil infiltration although little is known about the function of sVAP-1 in this model. Experiments using a pericyte-specific VAP-1 knockout mouse should provide more detailed information; however, a truly specific marker for pericytes has not yet been found.

There are some limitations in the present study. I cannot exclude off-target

effects related to the use of RTU-1096. The most plausible off-targets are MAO-A and MAO-B, which belong to the same enzyme class (monoamine oxidase) as VAP-1. Considering the very high specificity of this drug as shown above, however, it is unlikely that suppressed renal IR injury was through these off-targets. Moreover, the precise mechanism with which VAP-1 inhibition ameliorates renal IR injury is still unclear. Further in vitro and in vivo studies are clearly warranted.

In conclusion, despite several limitations, the present study suggests that VAP-1, which is expressed in pericytes of the kidney, controls neutrophil infiltration in renal IR injury, potentially allowing VAP-1 inhibition to be used as a novel therapeutic target in ischemic AKI.

ACKNOWLEDGEMENTS

I would like to express gratitude to Prof. Nangaku and Dr. Tanaka for providing me with numerous valuable opportunities and advice.

REFERENCES

[1] A. Lassnigg, D. Schmidlin, M. Mouhieddine, L. M. Bachmann, W. Druml, P. Bauer, *et al.*, "Minimal changes of serum creatinine predict prognosis in patients after cardiothoracic surgery: a prospective cohort study," *J Am Soc Nephrol,* vol. 15, pp. 1597-605, Jun 2004.

[2] N. Srisawat, E. E. Hoste, and J. A. Kellum, "Modern classification of acute kidney injury," *Blood Purif,* vol. 29, pp. 300-7, 2010.

[3] S. Tanaka, T. Tanaka, and M. Nangaku, "Hypoxia as a key player in the AKI-to-CKD transition," *Am J Physiol Renal Physiol,* vol. 307, pp. F1187-95, Dec 2014.

[4] S. G. Coca, S. Singanamala, and C. R. Parikh, "Chronic kidney disease after acute kidney injury: a systematic review and meta-analysis," *Kidney Int,* vol. 81, pp. 442-8, Mar 2012.

[5] N. H. Lameire, A. Bagga, D. Cruz, J. De Maeseneer, Z. Endre, J. A. Kellum, *et al.*, "Acute kidney injury: an increasing global concern," *Lancet,* vol. 382, pp. 170-9, Jul 2013.

[6] H. K. Eltzschig and T. Eckle, "Ischemia and reperfusion--from mechanism to translation," *Nat Med,* vol. 17, pp. 1391-401, 2011.

[7] L. Li and M. D. Okusa, "Macrophages, dendritic cells, and kidney ischemia-reperfusion injury," *Semin Nephrol,* vol. 30, pp. 268-77, May 2010.

[8] K. Singbartl and K. Ley, "Leukocyte recruitment and acute renal failure," *J Mol Med (Berl),* vol. 82, pp. 91-101, Feb 2004.

[9] H. Rabb, C. C. Mendiola, J. Dietz, S. R. Saba, T. B. Issekutz, F. Abanilla, *et al.*, "Role of CD11a and CD11b in ischemic acute renal failure in rats," *Am J Physiol,* vol. 267, pp. F1052-8, Dec 1994.

[10] K. J. Kelly, W. W. Williams, R. B. Colvin, and J. V. Bonventre, "Antibody to intercellular adhesion molecule 1 protects the kidney against ischemic injury," *Proc Natl Acad Sci U S A,* vol. 91, pp. 812-6, Jan 1994.

[11] H. Rabb, C. C. Mendiola, S. R. Saba, J. R. Dietz, C. W. Smith, J. V. Bonventre, *et al.*, "Antibodies to ICAM-1 protect kidneys in severe ischemic reperfusion injury," *Biochem Biophys Res Commun,* vol. 211, pp. 67-73, Jun 1995.

[12] T. Nemoto, M. J. Burne, F. Daniels, M. P. O'Donnell, J. Crosson, K. Berens, *et al.*, "Small molecule selectin ligand inhibition improves outcome in ischemic acute renal failure," *Kidney Int,* vol. 60, pp. 2205-14, Dec 2001.

[13] K. Salmela, L. Wramner, H. Ekberg, I. Hauser, O. Bentdal, L. E. Lins, *et al.*, "A randomized multicenter trial of the anti-ICAM-1 monoclonal antibody (enlimomab) for the prevention of acute rejection and delayed onset of graft function in cadaveric

renal transplantation: a report of the European Anti-ICAM-1 Renal Transplant Study Group," *Transplantation,* vol. 67, pp. 729-36, Mar 1999.

[14] R. Pannecoeck, D. Serruys, L. Benmeridja, J. R. Delanghe, N. Geel, R. Speeckaert*, et al.*, "Vascular adhesion protein-1: Role in human pathology and application as a biomarker," *Crit Rev Clin Lab Sci,* vol. 52, pp. 284-300, Dec 2015.

[15] M. Salmi and S. Jalkanen, "VAP-1: an adhesin and an enzyme," *Trends Immunol,* vol. 22, pp. 211-6, Apr 2001.

[16] S. Kaitaniemi, K. Grön, H. Elovaara, M. Salmi, S. Jalkanen, and K. Elima, "Functional modulation of vascular adhesion protein-1 by a novel splice variant," *PLoS One,* vol. 8, p. e54151, 2013.

[17] T. Noonan, S. Lukas, G. W. Peet, J. Pelletier, M. Panzenbeck, A. Hanidu*, et al.*, "The oxidase activity of vascular adhesion protein-1 (VAP-1) is essential for function," *Am J Clin Exp Immunol,* vol. 2, pp. 172-85, 2013.

[18] M. Salmi and S. Jalkanen, "A 90-kilodalton endothelial cell molecule mediating lymphocyte binding in humans," *Science,* vol. 257, pp. 1407-9, Sep 1992.

[19] M. Salmi, K. Kalimo, and S. Jalkanen, "Induction and function of vascular adhesion protein-1 at sites of inflammation," *J Exp Med,* vol. 178, pp. 2255-60, Dec 1993.

[20] K. Jaakkola, K. Kaunismäki, S. Tohka, G. Yegutkin, E. Vänttinen, T. Havia*, et al.*, "Human vascular adhesion protein-1 in smooth muscle cells," *Am J Pathol,* vol. 155, pp. 1953-65, Dec 1999.

[21] D. J. Smith and P. J. Vainio, "Targeting vascular adhesion protein-1 to treat autoimmune and inflammatory diseases," *Ann N Y Acad Sci,* vol. 1110, pp. 382-8, Sep 2007.

[22] C. M. Stolen, F. Marttila-Ichihara, K. Koskinen, G. G. Yegutkin, R. Turja, P. Bono*, et al.*, "Absence of the endothelial oxidase AOC3 leads to abnormal leukocyte traffic in vivo," *Immunity,* vol. 22, pp. 105-15, Jan 2005.

[23] J. Kiss, S. Jalkanen, F. Fülöp, T. Savunen, and M. Salmi, "Ischemia-reperfusion injury is attenuated in VAP-1-deficient mice and by VAP-1 inhibitors," *Eur J Immunol,* vol. 38, pp. 3041-9, Nov 2008.

[24] L. M. Salter-Cid, E. Wang, A. M. O'Rourke, A. Miller, H. Gao, L. Huang*, et al.*, "Anti-inflammatory effects of inhibiting the amine oxidase activity of semicarbazide-sensitive amine oxidase," *J Pharmacol Exp Ther,* vol. 315, pp. 553-62, Nov 2005.

[25] C. J. Weston, E. L. Shepherd, L. C. Claridge, P. Rantakari, S. M. Curbishley, J. W. Tomlinson*, et al.*, "Vascular adhesion protein-1 promotes liver inflammation and drives hepatic fibrosis," *J Clin Invest,* vol. 125, pp. 501-20, Feb 2015.

[26] H. C. Schilter, A. Collison, R. C. Russo, J. S. Foot, T. T. Yow, A. T. Vieira*, et al.*,

"Effects of an anti-inflammatory VAP-1/SSAO inhibitor, PXS-4728A, on pulmonary neutrophil migration," *Respir Res,* vol. 16, p. 42, 2015.

[27] A. M. O'Rourke, E. Y. Wang, A. Miller, E. M. Podar, K. Scheyhing, L. Huang*, et al.*, "Anti-inflammatory effects of LJP 1586 [Z-3-fluoro-2-(4-methoxybenzyl)allylamine hydrochloride], an amine-based inhibitor of semicarbazide-sensitive amine oxidase activity," *J Pharmacol Exp Ther,* vol. 324, pp. 867-75, Feb 2008.

[28] K. Noda, S. Miyahara, T. Nakazawa, L. Almulki, S. Nakao, T. Hisatomi*, et al.*, "Inhibition of vascular adhesion protein-1 suppresses endotoxin-induced uveitis," *FASEB J,* vol. 22, pp. 1094-103, Apr 2008.

[29] H. Xu, F. D. Testai, T. Valyi-Nagy, M. N Pavuluri, F. Zhai, D. Nanegrungsunk*, et al.*, "VAP-1 blockade prevents subarachnoid hemorrhage-associated cerebrovascular dilating dysfunction via repression of a neutrophil recruitment-related mechanism," *Brain Res,* vol. 1603, pp. 141-9, Apr 2015.

[30] H. L. Xu, M. Garcia, F. Testai, F. Vetri, A. Barabanova, D. A. Pelligrino*, et al.*, "Pharmacologic blockade of vascular adhesion protein-1 lessens neurologic dysfunction in rats subjected to subarachnoid hemorrhage," *Brain Res,* vol. 1586, pp. 83-9, Oct 2014.

[31] H. L. Xu, L. Salter-Cid, M. D. Linnik, E. Y. Wang, C. Paisansathan, and D. A. Pelligrino, "Vascular adhesion protein-1 plays an important role in postischemic inflammation and neuropathology in diabetic, estrogen-treated ovariectomized female rats subjected to transient forebrain ischemia," *J Pharmacol Exp Ther,* vol. 317, pp. 19-29, Apr 2006.

[32] J. Watcharotayangul, L. Mao, H. Xu, F. Vetri, V. L. Baughman, C. Paisansathan*, et al.*, "Post-ischemic vascular adhesion protein-1 inhibition provides neuroprotection in a rat temporary middle cerebral artery occlusion model," *J Neurochem,* vol. 123 Suppl 2, pp. 116-24, Nov 2012.

[33] P. H. Yu and D. M. Zuo, "Oxidative deamination of methylamine by semicarbazide-sensitive amine oxidase leads to cytotoxic damage in endothelial cells. Possible consequences for diabetes," *Diabetes,* vol. 42, pp. 594-603, Apr 1993.

[34] P. Jablonski, B. O. Howden, D. A. Rae, C. S. Birrell, V. C. Marshall, and J. Tange, "An experimental model for assessment of renal recovery from warm ischemia," *Transplantation,* vol. 35, pp. 198-204, Mar 1983.

[35] J. V. Bonventre, "Kidney injury molecule-1: a translational journey," *Trans Am Clin Climatol Assoc,* vol. 125, pp. 293-9; discussion 299, 2014.

[36] S. Arai, K. Kitada, T. Yamazaki, R. Takai, X. Zhang, Y. Tsugawa*, et al.*, "Apoptosis inhibitor of macrophage protein enhances intraluminal debris clearance and ameliorates acute kidney injury in mice," *Nat Med,* Jan 2016.

[37] G. Schley, B. Klanke, J. Schödel, F. Forstreuter, D. Shukla, A. Kurtz*, et al.*, "Hypoxia-inducible transcription factors stabilization in the thick ascending limb protects against ischemic acute kidney injury," *J Am Soc Nephrol,* vol. 22, pp. 2004-15, Nov 2011.

[38] S. Nourshargh and R. Alon, "Leukocyte migration into inflamed tissues," *Immunity,* vol. 41, pp. 694-707, Nov 2014.

[39] C. E. Ayres-Sander, H. Lauridsen, C. L. Maier, P. Sava, J. S. Pober, and A. L. Gonzalez, "Transendothelial migration enables subsequent transmigration of neutrophils through underlying pericytes," *PLoS One,* vol. 8, p. e60025, 2013.

[40] K. Stark, A. Eckart, S. Haidari, A. Tirniceriu, M. Lorenz, M. L. von Brühl*, et al.*, "Capillary and arteriolar pericytes attract innate leukocytes exiting through venules and 'instruct' them with pattern-recognition and motility programs," *Nat Immunol,* vol. 14, pp. 41-51, Jan 2013.

[41] A. C. Chung and H. Y. Lan, "Chemokines in renal injury," *J Am Soc Nephrol,* vol. 22, pp. 802-9, May 2011.

[42] M. Miura, X. Fu, Q. W. Zhang, D. G. Remick, and R. L. Fairchild, "Neutralization of Gro alpha and macrophage inflammatory protein-2 attenuates renal ischemia/reperfusion injury," *Am J Pathol,* vol. 159, pp. 2137-45, Dec 2001.

[43] D. Cugini, N. Azzollini, E. Gagliardini, P. Cassis, R. Bertini, F. Colotta*, et al.*, "Inhibition of the chemokine receptor CXCR2 prevents kidney graft function deterioration due to ischemia/reperfusion," *Kidney Int,* vol. 67, pp. 1753-61, May 2005.

[44] L. Li, L. Huang, A. L. Vergis, H. Ye, A. Bajwa, V. Narayan*, et al.*, "IL-17 produced by neutrophils regulates IFN-gamma-mediated neutrophil migration in mouse kidney ischemia-reperfusion injury," *J Clin Invest,* vol. 120, pp. 331-42, Jan 2010.