Online Supplement

Endothelial Krüppel-like factor 4 mediates the protective effect of statins against ischemic acute kidney injury

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Materials and Methods

Generation of endothelial *Klf4*-deficient mice

Animal protocols were approved by Keio University Animal Care and Use Committee. *Klf4*$^{loxP/loxP}$ mice, kindly provided by Dr. Klaus H. Kaestner[1], were bred with *Tek-Cre*$^{+/-}$ mice[2] to generate *Tek-Cre*$^{+/-}$/*Klf4*$^{loxP/+}$ mice. *Tek-Cre*$^{+/-}$/*Klf4*$^{loxP/+}$ mice were then bred with *Klf4*$^{loxP/loxP}$ mice to generate *Tek-Cre*$^{+/-}$/*Klf4*$^{loxP/loxP}$ (*Klf4* conditional knockout; *Klf4* cKO) mice and *Tek-Cre*$^{-/-}$/*Klf4*$^{loxP/loxP}$ (control) mice. Both mice were on the C57BL/6J background, and littermates were used for all comparisons. Genotyping was performed by PCR as described previously[3]. Five to six mice per each genotype and per each treatment were analyzed.

Ischemic AKI model

Male *Klf4* cKO and control mice at 12 to 14 weeks of age were allowed free access to water and standard mouse chow. Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Kidneys were exposed through flank incisions. Mice were subjected to 35 min of bilateral renal ischemia or sham surgery. Ischemia was induced by clamping both renal pedicles with non-traumatic microvessel clamps. The incisions were temporarily closed during ischemia or sham surgery. After the clamps were removed, reperfusion of the kidneys was visually confirmed. Some mice were treated with fluvastatin (20 mg/kg/day, Wako Pure Chemical, Osaka, Japan) by gavage for 3 days before surgery. Twenty-four hours after reperfusion, the mice were sacrificed under pentobarbital anesthesia, and blood samples as well as the kidneys were harvested.

Serum urea nitrogen and creatinine

The serum concentration of urea nitrogen was determined by the urease-indophenol method (Wako Pure Chemical). Serum creatinine concentrations were measured by an enzymatic method (Wako Pure Chemical).

Histology and Immunohistochemistry

The kidneys were fixed in 4% paraformaldehyde and embedded into paraffin. The 5-μm sections were prepared and subjected to hematoxylin-eosin staining and immunohistochemistry. Histological analyses were performed in a blind manner using an arbitrary scale, as previously described[4]. Proteinaceous casts and tubular necrosis were graded as follows: 0 (no damage), 1 (patchy isolated damage), 2 (damage less than 25%), 3 (damage between 25 and 50%), and 4 (more than 50% damage). The degree of medullary congestion was defined as follows: 0 (no vascular congestion), 1 (congestion with identification of erythrocytes by x400 magnification), 2 (congestion with identification of erythrocytes by x200 magnification), 3 (congestion with identification of erythrocytes by x100 magnification), and 4 (congestion with identification of erythrocytes by x40 magnification).

Immunohistochemistry was performed with antibodies for Klf4[5], neutrophil (7/4, Abcam, Cambridge, MA), CD3ε (M20, Santa Cruz Biotechnology, Santa Cruz, CA), Ly6c (ER-MP20, Abcam), and F4/80 (CI:A3-1, Abcam). Staining was visualized by diaminobenzidine, and sections were counterstained by hematoxylin.

Cell culture

HUVECs (Japanese Collection of Research Bioresources, Osaka, Japan) were cultured in MCDB107 medium supplemented with endothelial cell growth supplement (Sigma, St. Louis, MO) and 10% fetal bovine serum (Life Technologies, Carlsbad, CA). One day after plating at 20000 cells/cm^2, *KLF4* siRNA[6] or scrambled siRNA were transfected into HUVECs using Lipofectamine RNAiMAX (Life Technologies). On the next day, HUVECs were treated with 10

ng/mL human TNFα (R&D Systems, Minneapolis, MN), 1 μmol/L fluvastatin, and/or 1 μmol/L simvastatin (Sigma) for additional 24 hours.

Real-time RT-PCR

Total RNA prepared from the kidneys or HUVECs was used for real-time RT-PCR. Primer and probe sequences were described previously[7-9].

Western blotting

Western blotting was performed as described previously[3, 7, 9]. Antibodies used were as follows: Hmox1 (HO-1-1, Abcam), Rock1 (EP786Y, Abcam), VCAM1 (H276, Santa Cruz Biotechnology), p65 (F6, Santa Cruz Biotechnology), phospho-p65 at serine 536 (93H1, Cell Signaling Technology, Danvers, MA), and GAPDH (6C5, Millipore, Billerica, MA).

Quantitative chromatin immunoprecipitation assays

Quantitative chromatin immunoprecipitation assays were performed using anti-p65 antibody as described previously[3, 7, 9]. Real-time PCR was performed to amplify the promoter region of the human *VCAM1* gene[7].

Statistical analyses

Data are presented as mean ± SEM. Statistical analyses were done by SigmaPlot/SigmaStat9 (Systat Software Inc, San Jose, CA). After confirming that the data passed the normality test for parametric analyses, two-way or three-way factorial ANOVA were performed with a *post hoc* Fisher protected least significant difference test. P values < 0.05 were considered significant.

References

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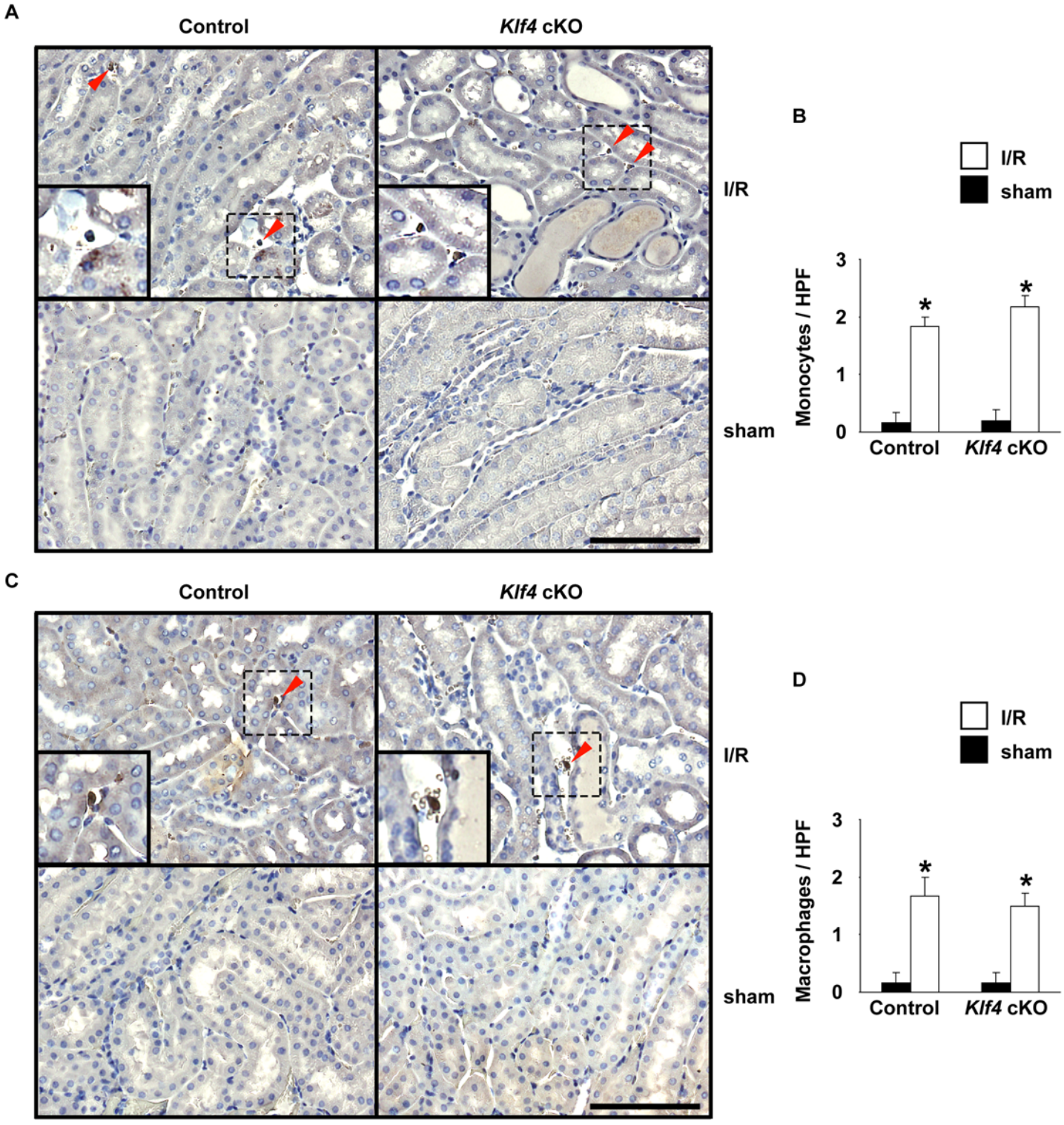

Supplemental Figure S1. Accumulation of monocytes/macrophages was not enhanced in *Klf4* cKO mice following renal I/R injury.

Klf4 cKO mice and control mice were subjected to bilateral renal ischemia for 35 min (I/R) or sham-operation, and the accumulation of monocytes (A and B) and macrophages (C and D) was examined 24 h after reperfusion. **A and C:** Representative pictures for immunohistochemical staining for monocytes (A) and macrophages (C) are shown. Monocytes (A) and macrophages (C) were visualized by diaminobenzidine, and sections were counterstained with hematoxylin. Bars: 100 μm. Red arrowheads indicate the positive cells. **Insets:** Dotted areas are enlarged. **B and D:** The numbers of monocytes (B) and macrophages (D) per 5 random fields in the kidneys were quantified. n=5-6 per each group. *P<0.05 compared with sham-operated mice.