

Online Data Supplement

Supplemental Methods

Reagents

Iron sucrose was obtained from Nang-Kuang Pharmaceutical Co. (Tainan, Taiwan). 2',7'-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester, apocynin, N-acetylcysteine, lucigenin, phosphatase inhibitor cocktails, and 3,3'-diaminobenzidine (DAB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Goat antibodies for intracellular cell adhesion molecule-1 and vascular cell adhesion molecule-1 were from R&D Systems (Minneapolis, MN, USA). Potassium ferrocyanide, hydrochloric acid, and neutral red for iron staining were from Muto Pure Chemicals (Tokyo, Japan).

Cell culture

Human aortic endothelial cells (HAECs; Cascade Biologics, Portland, OR, USA) were grown in Medium 200 (Cascade Biologics) supplemented with low-serum growth supplement (Cascade Biologics) in an atmosphere of 95% air and 5% CO₂ at 37°C in plastic flasks. The final concentrations of the components in Medium 200 were 2% fetal bovine serum, 1 µg/mL hydrocortisone, 10 ng/mL human epidermal growth factor, 3 ng/mL human fibroblast growth factor, 10 µg/mL heparin, and 1% antibiotic-antimycotic mixture (penicillin, streptomycin, and amphotericin B [Fungizone]) (Gibco BRL, Carlsbad, CA, USA). At confluence, the cells were subcultured at a 1:3 ratio and used at passage numbers 3–8.

U937, a human monocytic cell line, was obtained from American Type Culture Collection (Rockville, MD, USA) and grown in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic mixture.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay for cell viability

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma

Chemical Co., St. Louis, MO, USA) was used to measure cell viability. The principle of this assay is that mitochondrial dehydrogenase in viable cells reduces MTT to a blue formazan. Briefly, cells were grown in 96-well plates and incubated with various concentrations of iron sucrose for 4 h. After washing HAECs with phosphate-buffered saline (PBS), 100 μ L of medium containing MTT (0.5 mg/mL) was added to each well for incubation at 37°C for 4 h. The medium was then carefully removed to avoid disturbing the formazan crystals that had formed. One hundred microliters of dimethyl sulfoxide, which solubilizes formazan crystals, was added to each well, and the absorbance of the solubilized blue formazan was read at 540 nm using a microplate reader (Multiskan Ex, Thermo Labsystems, Beverly, MA). Dimethyl sulfoxide was the blank. The reduction in optical density caused by the drug was used as a measurement of cell viability. Optical density in each iron sucrose-treated group was normalized to the optical density of the cells incubated in the control medium, which were considered 100% viable (the untreated group).

***In vitro* mononuclear–endothelial cell adhesion assay**

To explore the effect of iron sucrose on leukocyte–endothelial cell adhesion, the adherence of U937 cells or human mononuclear cells (MNCs) to HAECs was examined under static conditions. Adhesion assays were then performed as previously described.¹ Briefly, U937 cells were labeled with 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (Sigma) at 37°C for 1 h, washed, and suspended in RPMI-1640. Confluent HAECs in 24-well plates were incubated with U937 cells (10^6 cells/mL) at 37°C for 1 h. Nonadherent leukocytes were removed, and plates were gently washed twice with PBS. Two hundred fifty microliters of cell lysis buffer (PBS containing 20% EtOH and 0.1% Tween 20) was added to each well. After centrifugation, the supernatant was transferred to measure the fluorescence intensity (relative fluorescence units) at 485 nm excitation and 530 nm emission using a fluorescence microplate reader (Victor II, Perkin Elmer, Waltham, MA, USA). The data were expressed as

the fold difference from the untreated control in each experiment.

Detection of intracellular reactive oxygen species production

The effect of iron sucrose on reactive oxygen species (ROS) production in HAECs was determined using fluorometric assay with 2',7'-dichlorofluorescein diacetate (Molecular Probe) as the probe.² Briefly, 15 μ M 2',7'-dichlorofluorescein diacetate was added to the medium, which was incubated for 20 min at 37°C. After washing with Hank's balanced salt solution (without Ca^{2+} or Mg^{2+}), 250 μ L cell lysis buffer (PBS containing 20% EtOH and 0.1% Tween 20) was added to each well. After centrifugation, the supernatant was transferred to measure the fluorescence intensity (relative fluorescence units) at 485 nm excitation and 530 nm emission using a fluorescence microplate reader (Victor II).

Western blot

Protein extracts were prepared as previously described.¹ Total protein was quantified using a Bio-Rad (Hercules, CA, USA) protein assay, and processed for western blot using primary goat antibodies for vascular cell adhesion molecule-1 and intracellular cell adhesion molecule-1. Afterward, species-directed secondary goat horseradish peroxidase-conjugated monoclonal antibodies were used. Anti- β -actin antibody was used as a loading control. Densitometric analysis using ImageQuant software (GE Healthcare, Piscataway, NJ, USA) was conducted to semiquantify the data.

Detection of intracellular ROS production of circulating mononuclear cells in mice

Circulating MNCs were isolated from heparinized mice blood by centrifugation at 400 g for 30 min at room temperature over Histopaque 1083 (Sigma). Isolated circulating MNCs were then resuspended with serum-free RPMI 1640 medium. ROS production of MNCs from mice was determined using fluorometric assay with 2',7'-dichlorofluorescein diacetate (Molecular Probe) as the probe.²

Iron histochemistry

Iron deposits on atherosclerotic lesions were examined using Perls' Prussian blue reaction with DAB intensification. Briefly, after Perls' reaction, sections were incubated with 0.5% DAB in 0.1 M phosphate buffer (pH 7.4) for 20 min, followed by 15 min in the same medium containing 0.005% H₂O₂. The reaction was stopped via rinsing in deionized H₂O for >30 min, and the sections were counterstained with hematoxylin. Negative control slides were prepared via DAB intensification without preincubation with Perl's solution.

Determination of serum iron, total iron binding capacity, and ferritin

Serum iron and total iron binding capacity (TIBC) were measured using the Iron/TIBC Reagent Set (BQ Kits). Serum ferritin was measured with an immunoassay kit (Abcam).

Isolation of peripheral blood mononuclear cells

Circulating MNCs from iron-treated CKD patients and healthy subjects were isolated and extracted via density ultracentrifugation with minor modification.¹ In brief, 10 mL peripheral venous blood was drawn into a VACUTAINER[®] CPT[™] tube (BD bioscience, San Jose, CA, USA) with 0.1 M sodium citrate at room temperature at bedside after 30 min of bed rest with the study subjects in a supine position. The tubes were gently inverted 10 times. The blood was centrifuged and washed with ethylenediaminetetraacetic acid/Hank's balanced salt solution per manufacturer instructions. Isolated circulating MNCs were then resuspended with serum-free RPMI 1640 medium for labeling. All samples used in these experiments had at least 95% viability of isolated MNCs assessed using trypan blue exclusion. The isolation procedures were carried out at room temperature within 2 h of blood collection. After density ultracentrifugation, the separated plasma was frozen and stored at -20°C until analysis of circulating levels of adhesion molecules.

Supplemental References

1. Kuo KL, Hung SC, Lin YP, Tang CF, Lee TS, Lin CP, Tarng DC: Intravenous ferric chloride hexahydrate supplementation induced endothelial dysfunction and increased cardiovascular risk among hemodialysis patients. *PLoS One* 7: e50295, 2012
2. Chen YH, Lin SJ, Chen JW, Ku HH, Chen YL: Magnolol attenuates VCAM-1 expression in vitro in TNF-alpha-treated human aortic endothelial cells and in vivo in the aorta of cholesterol-fed rabbits. *Br J Pharmacol* 135: 37–47, 2002

Supplemental Table 1. Effect of iron supplementation on serum iron parameters for sham or subtotal nephrectomized wild-type mice treated with saline or iron sucrose via intraperitoneal administration

	Sham		SNx	
	Iron (-) (n = 10)	Iron (+) (n = 10)	Iron (-) (n = 10)	Iron (+) (n = 10)
Serum iron (µg/dL)	364 ± 25	523 ± 80*	413 ± 23	644 ± 91*
TIBC (µg/dL)	588 ± 50	688 ± 142	732 ± 49	759 ± 128
Serum ferritin (µg/L)	40 ± 4	54 ± 2*	48 ± 6	56 ± 5*

SNx, subtotal nephrectomized; TIBC, total iron-binding capacity. Data are mean ± SD.

* $P < 0.05$ compared with non-iron-treated sham or SNx group, respectively

Supplemental Table 2. Effect of intravenous iron sucrose on serum iron parameters in CKD patients as compared with healthy controls and CKD patients not treated with iron sucrose

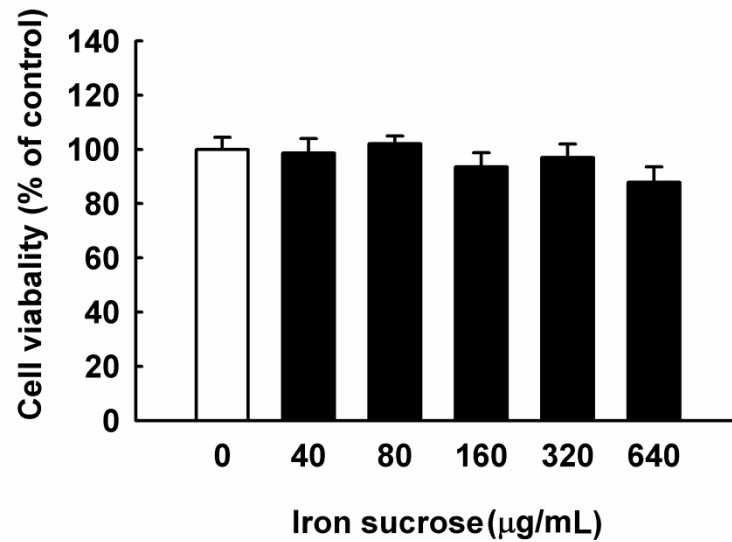
	Controls (n = 20)	CKD (n = 20)	CKD + iron (n = 20)
Serum iron (µg/dL)	43 ± 12	73 ± 20 ^a	90 ± 18 ^{a,b}
TIBC (µg/dL)	218 ± 29	216 ± 34	216 ± 42
Serum ferritin (µg/L)	74 ± 42	142 ± 95.8 ^a	516 ± 164 ^{a,b}

CKD, chronic kidney disease; TIBC, total iron-binding capacity. Data are mean ± SD.

^a*P* < 0.05 compared with Controls

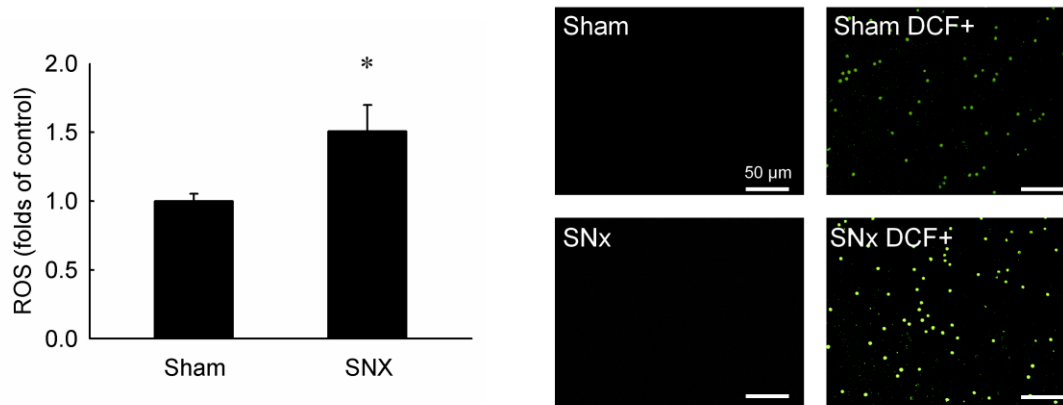
^b*P* < 0.05 compared with CKD not treated with iron sucrose

Supplemental Figure 1



Supplemental Figure 1. Cell viability of human aortic endothelial cells 24 h after culture with various concentrations of iron sucrose determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. The percentage of cell viability in the iron sucrose-treated groups was compared with that in the untreated group (cell viability = 100%). The data are expressed as the mean \pm SEM from 3 independent experiments.

Supplemental Figure 2



Supplemental Figure 2. Comparison of ROS production in circulating mononuclear cells between sham or subtotal nephrectomized wild-type mice. Circulating mononuclear cells were isolated for measurement of ROS production at 8 weeks after sham or subtotal nephrectomy. Value are expressed as mean \pm SEM. Scale bar represents 50 μ m. * P < 0.05, compared with sham group, n = 10 in each group. Abbreviations: DCF, 2',7'-dichlorofluorescein; ROS, reactive oxygen species, SNx, subtotal nephrectomized.