Dietary iron restriction alleviates renal tubulointerstitial injury induced by protein overload in mice

Yasumasa Ikeda1, Yuya Horinouchi1, Hirofumi Hamano1,2, Tasuku Hirayama3, Seiji Kishi4, Yuki Izawa-Ishizawa1, Masaki Imanishi2, Yoshito Zamami2,5, Kenshi Takechi6, Licht Miyamoto7, Keisuke Ishizawa2,5, Ken-ichi Aihara6, Hideko Nagasawa3, Koichiro Tsuchiya7 & Toshiaki Tamaki1

Increased proteinuria causes tubulointerstitial injury due to inflammation in chronic kidney disease (CKD). Iron restriction exhibits protective effects against renal dysfunction; however, its effects against protein overload-induced tubulointerstitial damage remain unclear. Here, we investigated dietary iron restriction effect on tubulointerstitial damage in mice with protein-overload tubulointerstitial injury. Renal tubulointerstitial injury in animal model was induced by intraperitoneal injection of an overdose of bovine serum albumin (BSA). We divided mice into three groups: normal saline + normal diet (ND), BSA + ND, and BSA + iron-restricted diet (IRD). BSA overload induced renal tubulointerstitial injury in the ND mice, which was ameliorated in the IRD mice. Inflammatory cytokines and extracellular matrix mRNA expression was upregulated in BSA + ND mice kidneys and was inhibited by IRD. BSA-induced increase in renal superoxide production, NADPH oxidase activity, and p22phox expression was diminished in the IRD mice. IRD suppression increased BSA-induced renal macrophage infiltration. Moreover, BSA mice exhibited nucleotide-binding oligomerisation domain-like receptor pyrin domain-containing protein (NLRP) inflammasome activation, which was inhibited by IRD. Ferrous iron increased in kidneys with BSA overload and was inhibited by IRD. Thus, iron restriction inhibited oxidative stress and inflammatory changes, contributing to the protective effect against BSA overload-induced tubulointerstitial injury.

The number of patients with chronic kidney disease (CKD) has been increasing worldwide, and the presence of CKD worsens morbidity and mortality. Proteinuria, including albuminuria, is a biomarker for CKD, is associated with renal tubular and tubulointerstitial damage, and causes further progression of kidney injury and deterioration of renal function1-3. Proteinuria induces tubulointerstitial injury through apoptosis4, inflammation4, epithelial–mesenchymal transition5, and oxidative stress6. Recent studies have demonstrated that nucleotide-binding oligomerisation domain-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome activation, which was inhibited by IRD. Ferrous iron increased in kidneys with BSA overload and was inhibited by IRD. Thus, iron restriction inhibited oxidative stress and inflammatory changes, contributing to the protective effect against BSA overload-induced tubulointerstitial injury.
iron metabolism and homeostasis \(^1\); therefore, iron is potentially involved in renal pathophysiology. In different experimental animal models, iron is involved in determining the pathological condition of CKD such as diabetic nephropathy \(^12–14\), hypertensive kidney injury \(^15, 16\), and renal fibrosis \(^17\), and these diseases are ameliorated by iron reduction in the body. Dietary iron restriction inhibits the increment of urinary albuminuria in db/db mice \(^12\); however, it remains unclear whether iron restriction can prevent protein overload-induced renal injury.

In the present study, we determined that dietary iron restriction alleviates protein overload-induced tubulointerstitial injury, and inhibits oxidative stress and inflammatory changes in mice. Moreover, ferrous iron production is elevated in the kidneys with protein overload, suggesting its involvement in the pathological condition of protein-overload renal injury through oxidative stress via the Fenton reaction.

**Results**

**Effects of dietary iron restriction on BSA-induced tubulointerstitial injury.** Renal tubulointerstitial injury was induced in mice with BSA overload, and it was alleviated by dietary iron restriction (Fig. 1a (upper) and b). Similar to histological analysis, the mRNA expression of lipocalin-2, a marker of tubulointerstitial damage, was also upregulated in BSA-overload mice, which was inhibited by iron-restricted diet (IRD, Fig. 1c). BSA-induced upregulation of fibrosis-related genes, such as collagen 1 and fibronectin, was also suppressed in mice with IRD (Fig. 1a (middle and lower), and d). These results suggested the favourable effect of dietary iron restriction in protein-overload tubulointerstitial injury.

**Dietary iron restriction attenuates the BSA-induced renal inflammatory changes.** BSA augmented renal inflammatory cytokines, including TNF-\(\alpha\), MCP-1, IL-6, and PAI-1, which were abolished by iron restriction (Fig. 2a). Increment of macrophage infiltration in the tubulointerstitium and increased F4/80 mRNA expression was observed in mice with BSA overload and was reduced by IRD (Fig. 2b–d).

**BSA-induced renal oxidative stress was reduced by iron restriction.** BSA promotes oxidative stress production through NADPH oxidase-dependent pathway \(^6\). We performed dihydroethidium (DHE) staining to assess oxidative stress production. BSA-induced renal oxidative stress in mice kidneys was reduced by IRD (Fig. 3a and b). The increase in NADPH oxidase activity was elevated in the BSA+ND group mice kidneys and...
Figure 2. Iron restriction prevents BSA-induced inflammatory changes. (a) Quantitative analysis of mRNA expression for inflammation-related genes in the kidneys of mice in each group. Values are expressed as mean ± SD. *P < 0.05, **P < 0.01, n = 6–9 in each group. (b) Representative figures of F4/80 immunohistochemical staining in the kidney. (c) Semiquantitative analysis of the F4/80-positive spot in the kidneys of mice in each group. Values are expressed as mean ± SD. *P < 0.05, **P < 0.01, n = 8 in each group. (d) Quantification of F4/80 mRNA in the kidneys of mice in each group. Values are expressed as mean ± SD. *P < 0.05, **P < 0.01, n = 8 in each group.

Figure 3. Inhibitory effect of iron restriction against BSA-induced oxidative stress in the kidney. (a) Representative images of dihydroethidium (DHE) staining in the kidneys of mice in each group. (b) Semiquantitative analysis of fluorescence intensity. Values are expressed as mean ± SD. *P < 0.05, **P < 0.01, n = 8 in each group. (c) NADPH activity in the kidney. Values are expressed as mean ± SD. *P < 0.05, **P < 0.01, n = 8 in each group. (d) p22phox mRNA expression in the kidneys of mice in each group. Values are expressed as mean ± SD. *P < 0.05, **P < 0.01, n = 7–9 in each group. (e) p22phox protein expression in the kidney. Upper panel, representative figures of p22phox and β-actin from the kidney (full-length blots are presented as Supplementary Figure 1); and lower panel, semiquantitative analysis of densitometry for p22phox expression. Values are expressed as mean ± SD. *P < 0.05, **P < 0.01, n = 10–12 in each group.
was inhibited by IRD (Fig. 3c). Both mRNA and protein expression of p22phox, a subunit of the NADPH oxidase complex, was augmented in the BSA + ND group mice kidneys and was inhibited in the BSA + IRD group mice (Fig. 3d).

**NLRP3 inflammasome activation was abolished by dietary iron restriction.** BSA + ND group mice exhibited upregulation of mRNA and protein expression of NLRP3, predominantly in the renal tubule, and this increase was diminished by IRD (Fig. 4a–d). Similarly, the expression of p10 caspase-1, ASC, and mature IL-1β was also upregulated by BSA overload and was inhibited by IRD (Fig. 4c and d).

**Changes in iron content and ferrous iron in the kidney.** Dietary iron restriction reduced renal iron content and induced mild anaemia in the mice with BSA overload. Although BSA overload did not alter the total iron content in the kidney compared to the vehicle group, renal ferrous iron was increased by BSA overload, and the increase was suppressed by IRD (Table 1 and Fig. 5).

**Alteration of renal function and iron-related parameters in the mice with BSA overload.** BSA overload resulted in an increase in the blood urea nitrogen (BUN) level, which was ameliorated by IRD. On the other hand, no change in the plasma creatinine level was observed between the three groups. Additionally, no change in the plasma ferritin level was observed between the mice administered with vehicle and the mice with BSA overload. Plasma iron and transferrin saturation (TSAT) were observed to be elevated in the BSA-treated mice. Plasma ferritin, plasma iron and TSAT levels were diminished in the BSA-treated mice with IRD. Plasma hepcidin-1 was also found to be elevated in the mice with BSA overload, which was dramatically reduced by IRD (Table 2).

**Discussion**
Proteinuria is an early biomarker for patients with CKD and a causal factor for the progression of CKD by eliciting tubular damage and tubulointerstitial injury. We first demonstrated that BSA overload enhanced ferrous iron levels in the kidney, although the renal total iron content was not altered. Renal iron content is increased in the mice with unilateral ureteral obstruction (UUO) surgery or in mice with 5/6 nephrectomy, indicating that the increment in ferrous iron is due to the increase of total iron content. Furthermore, dietary iron reduction
prevented the progression of diabetic nephropathy in the db/db mice, however, no difference was observed in the renal iron content of the control mice and that of the db/db mice\(^{12}\), suggesting the involvement of the labile ferrous iron, rather than the total iron content, in the pathological condition. In cisplatin-induced nephropathy and ischemia-reperfusion injury, bleomycin-detectable iron (iron capable of catalysing the reaction of free radicals is same as ferrous iron) and hydroxyl radical formation increased in the mice kidneys\(^{18, 19}\). It has been clinically observed that urinary catalytic iron increased during diabetic nephropathy or glomerulonephritis\(^{20}\). Thus, ferrous iron increased in these kidney diseases, and may be responsible for their pathological condition. Although the primary mechanism of ferrous iron augmentation remains unclear, labile ferrous iron is potentially involved.

### Table 1.

|                          | Vehicle  | BSA       | BSA + IRD |
|--------------------------|----------|-----------|-----------|
| Body weight (g)          | 23.4 ± 1.1 | 23.7 ± 1.7 | 24 ± 2 |
| Right kidney weight (mg) | 109 ± 7 | 131 ± 18** | 129 ± 10** |
| Right kidney weight to body weight ratio | 4.6 ± 0.2 | 5.5 ± 0.5** | 5.4 ± 0.2* |
| Renal iron content (µg/g tissue) | 9.9 ± 3.6 | 9.7 ± 3 | 5.4 ± 1.9** |
| Red blood cell (×10\(^3\)/µL) | 827 ± 63 | 829 ± 48 | 760 ± 49** |
| Haemoglobin (g/dL)    | 12.6 ± 1 | 12.4 ± 0.7 | 10.6 ± 1.1** |
| Haematocrit (%)         | 38.1 ± 3.3 | 38.1 ± 2 | 32.1 ± 3.1*** |
| MCV (fL)                | 46.1 ± 1 | 45.8 ± 1.1 | 42.4 ± 1.8*** |
| MCH (pg)                | 15.2 ± 3.3 | 15 ± 0.2 | 14 ± 0.6*** |
| MCHC (g/dL)             | 33 ± 0.7 | 32.6 ± 0.4 | 33.3 ± 0.4# |

Data represent mean ± SD; \( n = 8 \) or \( 9; \) *\( P < 0.05 \), **\( P < 0.01 \) vs. control mice; *\( P < 0.05 \), **\( P < 0.01 \) vs. BSA-treated mice.

**Figure 5.** The effect of BSA overload on ferrous iron in the kidney. Upper panels, representative figures of RhoNox-1 staining in kidneys in each group; lower panel, semiquantitative analysis of RhoNox-1 staining of kidneys in each group. Values are expressed as mean ± SD. **\( P < 0.01 \), \( n = 4 \) in each group.**
fibrosis in CKD such as diabetic nephropathy, 5/6 nephrectomy, and UUO-induced renal fibrosis. This supported the findings of our present study, since iron was essential for macrophage infiltration, inflammatory cytokines, and oxidative stress. As expected, renal superoxide and labile iron levels were reduced by IRD. Therefore, protein overload causes oxidative stress through the iron-dependent Fenton reaction and NADPH oxidase activation in the kidney. Therefore, iron restriction diminished NADPH oxidase activity and p22phox expression in diabetic nephropathy and renal fibrosis induced by unilateral ureteral obstruction. This supported the findings of our present study, since iron was essential for enzymatic activity of NADPH oxidase and biosynthesis of p22phox subunit, which is a haemprotein.

Therefore, protein overload-induced increment in renal ferrous iron suggested the promotion of oxidative stress via the Fenton reaction. As expected, renal superoxide and labile iron levels were reduced by IRD. Similar to this study, it has been observed that renal injuries induced by cisplatin or ischemia–reperfusion were diminished by iron chelation, through the inhibition of hydroxyl radical production, resulting in a decrease in the ferrous iron levels. In addition to the Fenton reaction, NADPH oxidase activity and p22phox expression was upregulated in the kidneys of mice with protein overload, which was inhibited by dietary iron restriction. Protein overload augments NADPH oxidase activity. We demonstrated that iron reduction diminished NADPH oxidase activity and p22phox expression in diabetic nephropathy and renal fibrosis induced by unilateral ureteral obstruction. This supported the findings of our present study, since iron was essential for enzymatic activity of NADPH oxidase and biosynthesis of p22phox subunit, which is a haemprotein.

Therefore, protein overload causes oxidative stress through the iron-dependent Fenton reaction and NADPH oxidase in the kidney, and these changes were inhibited by iron reduction, contributing to the alleviation of BSA-induced tubulointerstitial injury.

Table 2. Effects of bovine serum albumin (BSA) treatment and iron-restricted diet (IRD) on the levels of plasma total protein, plasma albumin, blood urea nitrogen (BUN), plasma creatinine, plasma ferritin, plasma iron, transferrin saturation (TSAT), and plasma hepcidin-1 in mice. Data represent mean ± SD; n = 5–13; *P < 0.05, **P < 0.01 vs. control mice; †P < 0.05, ‡P < 0.01 vs. BSA-treated mice.

|                  | Vehicle | BSA | BSA + IRD |
|------------------|---------|-----|-----------|
| Plasma total protein (g/dL) | 6.3 ± 1.2 | 9.2 ± 1.1** | 9.2 ± 0.9** |
| Plasma albumin (g/dL) | 2.8 ± 0.4 | 5.8 ± 0.9** | 5.6 ± 1.1** |
| BUN (mg/dL)      | 29.2 ± 5.0 | 40.0 ± 9.8** | 32.4 ± 5.7* |
| Plasma creatinine (mg/dL) | 0.61 ± 0.08 | 0.61 ± 0.09 | 0.59 ± 0.09 |
| Plasma ferritin (ng/mL) | 814.2 ± 178.6 | 631.8 ± 170.0 | 323.2 ± 145.9** |
| Plasma iron (μg/dL) | 60.0 ± 7.3 | 78.2 ± 9.6** | 61.9 ± 12.1** |
| TSAT (%)         | 25.1 ± 5.74 | 38.5 ± 6.8** | 25.8 ± 8.3** |
| Plasma hepcidin-1 (ng/dL) | 51.0 ± 33.8 | 112.6 ± 30.8** | 0.4 ± 0.11*** |

*P < 0.05, **P < 0.01 vs. control mice; †P < 0.05, ‡P < 0.01 vs. BSA-treated mice.
factor 23 (FGF23), a bone-derived phosphaturic hormone. The level of FGF23 is elevated in patients with CKD. Such elevated FGF23 levels are independently associated with the progression of CKD, cardiovascular events, and mortality\(^3\). Therefore, the beneficial effects of IRD on CKD might be offset by an increase in the level of FGF23. Further investigation is required to elucidate the beneficial and adverse effects of iron restriction on CKD.

In conclusion, augmentation of ferrous iron induced by protein overload causes oxidative stress via the Fenton reaction and NADPH oxidase, resulting in renal tubulointerstitial injury and inflammatory changes, which are ameliorated by iron restriction. These observations indicate that iron-mediated oxidative stress results in the pathogenesis of protein overload-induced renal injury.

Materials and Methods

Materials. We purchased and used the following commercially available antibodies: anti-interleukin-1β, anti-ASC (apoptosis-associated speck-like protein containing a CARD), anti-caspase-1 (p10), anti-fibronectin (Santa Cruz Biotechnology, Inc., Dallas, TX), anti-NLRP3 (Cell Signalling Technology, Danvers, MA), anti-F4/80 (Bio-Rad Laboratories, Hercules, CA), anti-type I collagen (SouthernBiotech, Birmingham, AL), and anti-5-actin (protein loading control, Cell Signalling Technology).

Animal preparation and procedure. All experimental procedures for mice were performed in accordance with the guidelines of the Animal Research Committee of Tokushima University Graduate School, and the protocol was approved by the Institutional Review Board of Tokushima University Graduate School for animal protection (Permit Number: 14137). The mice were randomly divided into three groups: vehicle with normal diet (ND)-fed group, BSA with ND-fed group, and BSA with IRD-fed group. Seven-week-old male C57BL/6J mice were obtained from Nippon CLEA (Tokyo, Japan) and were maintained with ad libitum access to water and food. Then, mice were either fed with commercially available ND [D03072502 (3.9 mg Fe/100 g food)] or IRD [D03072501 (0.3 mg Fe/100 g food)] (Research Diets, Inc. New Brunswick, NJ). After 1 week, the mice were intraperitoneally injected with BSA. A stepwise increment dose regime of BSA was employed for inducing protein overload tubulointerstitial injury in the mice\(^7\). In brief, the initial BSA dose was 2 mg/g body weight on the first day and was increased gradually to the maximum dose of 10 mg/g body weight on day 5, which was maintained for 7 days. For the vehicle-treated group, the mice received intraperitoneal injection of normal saline. On day 12, 24 h after the last BSA dose, the mice were euthanised by intraperitoneally injecting an overdose of pentobarbital, and kidneys were removed and stored at −80°C until further use.

Preparation of BSA solution. BSA (Cohn fraction V (019–23293); Wako Pure Chemical Industries, Ltd., Osaka, Japan) was dissolved in normal saline, and the final concentration was 330 mg/mL. The concentration of endotoxin in BSA solution was 0.03 ng/mL, as detected by the limulus amaebocyte lysate assay (Pierce™ LAL Chromogenic Endotoxin Quantitation Kit, Thermo Fisher Scientific, Inc., Waltham, MA).

Real-time PCR for mRNA quantification. RNA extraction, cDNA synthesis, and quantitative RT-PCR methods have been described previously in detail\(^12\). In brief, the tissues were homogenised in the RNAiso reagent (TAKARA Bio, Inc., Otsu, Japan) using a Minilys tissue homogeniser (Bertin Instruments, Montigny-le-Bretonneux, France). RNA extraction and cDNA synthesis were performed according to the manufacturer’s instructions [PrimeScript RT reagent kit with gDNA Eraser (Perfect Real Time); TAKARA Bio, Inc.]. Quantitative RT-PCR was performed using the CFX Connect Real-Time PCR Detection System (Bio-RAD Laboratories Inc., Hercules, CA, USA) with THUNDERBIRD SYBR qPCR Mix (TOYOBO CO., LTD., Osaka, Japan). The primer sets used were as follows: 5′-ACGGGATGATCTAAGAAG-3′ and 5′-GTGGTGGAGGACGACGTAGT-3′ for TNF-α, 5′-GGAGCTCTAGATGAGCAA-3′ and 5′-GACCAAGGGAAAGATTACA-3′ for monocyte chemotactic protein-1 (MCP-1), 5′-CCGGAGAGAGGTACCTCACAG-3′ and 5′-TCCAGATTCTCCAGAAGAC-3′ for IL-6, 5′-GGAGGGAGCTCTCTGATCG-3′ and 5′-GTTCCGGCGTCTGATCAGT-3′ for collagen-1, 5′-GCCCTTCCGAGCAAGCAG-3′ and 5′-GACAAAGGCCTGAGGAGGA-3′ for PAI-1, 5′-ACAGACGTCACCTCCCTGA-3′ and 5′-TGTGACTCTGTTTCTC-3′ for fibronectin, 5′-CTGTAACCGGATGAGCAAAC-3′ and 5′-CTGTAACCGGATGAGCAAAC-3′ for F4/80, 5′-GGAGCTCTCCCTTATTAGC-3′ and 5′-CTGCCCTTTCACCTCCTC-3′ for p22phox, 5′-GGAGACCAAGGATGCTG-3′ and 5′-GGGAGACCAAGGATGCTG-3′ for lipocalin-2,

Protein extraction and western blot analysis. Protein preparation and western blotting were performed as previously described\(^12\). In brief, kidney tissue samples were homogenised with the Minilys homogeniser (Bertin Instruments, Montigny-le-Bretonneux, France) and proteins were extracted. The extracted proteins were boiled for 5 min in the Laemmli sample buffer and separated using SDS–PAGE. Proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane and the membrane was blocked for 1 h at room temperature. Next, the membrane was incubated individually with each primary antibody overnight at 4°C, followed by incubation for 1 h with the secondary antibody. Then, immunoreactive bands were detected using a chemiluminescent reagent and visualised by exposure onto an X-ray film or by C-DiGit Blot Scanner (LI–COR, Lincoln, Nebraska, USA). Densitometry of the visualised bands was quantified using the Image J 1.38x software (https://imagej.nih.gov/ij/).
Histological analysis for tubulointerstitial damage. The assessment of renal tubulointerstitial damage has been described previously. In brief, the kidney tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin. Samples were cut into 3-µm sections, and the sections were stained with haematoxylin and eosin (HE). Tubular injuries were scored in a blinded manner according to the percentage of damage (including atrophy, flattening of the proximal tubule epithelial cells, and tubular dilation) as follows: 0, normal; 1, < 20%; 2, 20–40%; 3, 40–60%; 4, 60–80%, and 5, > 80%. Ten random microscopic fields per section were used for quantification.

Immunohistochemistry of the kidney samples. Immunohistochemical staining of F4/80 and fibronectin was performed as described previously. In brief, the paraffin-embedded kidney samples were cut into 3-µm sections, deparaffinised, processed for antigen retrieval using 10 mM citrate buffer at 95 °C for 20 min, and cooled for 20 min. For collagen-1 staining, the frozen tissue sections were used. Non-fixed kidney tissues were embedded in the Tissue-Tek O.C.T. Compound (Sakura Finetek, Tokyo, Japan) and subjected to snap freezing using the liquid nitrogen–cold isopentane mixture. Samples were cut into 8-µm sections, dried, and fixed in 10% neutral formaldehyde for 10 min. After blocking, the sections were incubated with the primary antibody at 4 °C overnight. Antibody distribution was visualised using a streptavidin–biotin complex assay and a 3,3′-diaminobenzidine (DAB) substrate kit (LSAB + Kit Universal; Dako Japan, Tokyo, Japan). Sections incubated without the primary antibody were used as negative controls. The evaluation of macrophage infiltration in tubulointerstitium was performed as described previously. Briefly, ten microscopic fields were randomly selected in the renal cortex and the macrophage-positive area was expressed as a percentage of the total area, except the areas under tubular lumen, glomeruli, and vessels, using the ImageJ 1.38x software.

Measurement of tissue iron content. Tissue iron content was measured using an iron assay kit according to the manufacturer’s instructions (Metallo assay; Metallogenics Co., Ltd., Chiba, Japan), as described previously. In brief, the kidney tissue samples were homogenised in the cell lysis buffer, and the non-centrifuged crude lysates with 0.05 M hydrochloric acid were mixed at regular intervals for 30 min, and centrifuged with 12,000 rpm for 10 min at 4 °C. Subsequently, the supernatants were used for assay. Tissue iron concentration was corrected using tissue weight and expressed as µg/g of wet tissue.

Measurement of NADPH oxidase activity. NADPH oxidase activity was measured as previously described. In brief, the kidney tissue sample was immediately homogenised in the NADPH oxidase lysis buffer and sonicated for 3 s. The NADPH substrate (final concentration, 300 µM) was added to the renal suspension with lucigenin (5 µM). Luminescence was measured every second for 60 min in a plate reader (SpectraMax Paradigm FilterMax F3; Molecular Devices Japan, Tokyo, Japan). NADPH activity was expressed as relative luminescence units normalised to the protein concentration.

In situ oxidative stress detection in the kidney tissue. Detection of superoxide production in the kidney was evaluated by DHE staining method as previously described. Non-frozen tissue sections were washed with PBS, incubated with DHE in PBS (10 µM) in a dark, humidified container at room temperature for 30 min, and observed using fluorescence microscopy.

Detection of renal ferrous iron. RhoNox-1 was used to detect ferrous iron content in the kidney. The staining method used has been described previously. First, frozen sections were washed three times in Hank’s balanced salt solution (HBSS) for 5 min, fixed in 10% neutral formaldehyde for 1 min, and washed three times with HBSS for 5 min. Next, the fixed sections were incubated with RhoNox-1 in HBSS (5 µM) in a dark, humidified container at room temperature for 30 min. After washing three times with HBSS, the sections were covered with a small drop of the mounting medium and observed using fluorescence microscopy. The section stained with RhoNox-1 was observed and quantified using fluorescence microscopy.

Haematological and biochemical analysis. Peripheral blood samples were analysed at Shikoku Chuken, K.K. (Kagawa, Japan). The plasma total protein concentration was measured by the bicinchoninic acid assay (BCA) assay. The analysis of plasma albumin, BUN, and TSAT was performed at the Nagahama Life Science Laboratory (Shiga, Japan). The plasma creatinine level was measured using the LabAssay Creatinine kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The plasma ferritin concentration was determined using the Mouse Ferritin ELISA kit (Immunochemistry Consultants Laboratory, Newberg, OR) according to the manufacturer’s instructions. The hepcidin concentration in mice was measured using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF–MS); Medical Care Proteomics Biotechnology Co., Ltd., Kanazawa, Japan).

Statistical analysis. Data are presented as mean ± standard deviation (SD). Significant differences between the three groups were determined using one-way ANOVA followed by Tukey’s post-hoc test. The differences between data were considered to be statistically significant at P < 0.05.

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