Dietary iron restriction inhibits progression of diabetic nephropathy in db/db mice

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1Department of Pharmacology, Institute of Health Biosciences, University of Tokushima Graduate School, Tokushima, Japan; 2Student Laboratory, Faculty of Medicine, University of Tokushima, Tokushima, Japan; 3Department of Medical Pharmacology, Institute of Health Biosciences, University of Tokushima Graduate School, Tokushima, Japan; and 4Division of Molecular Pharmacology, Tottori University, Tottori, Japan

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Ikeda Y, Enomoto H, Tajima S, Izawa-Ishizawa Y, Kihira Y, Ishizawa K, Tomita S, Tsuchiya K, Tamaki T. Dietary iron restriction inhibits progression of diabetic nephropathy in db/db mice. Am J Physiol Renal Physiol 304: F1028–F1036, 2013. First published February 6, 2013; doi:10.1152/ajprenal.00473.2012.—Excess iron causes oxidative stress through hydroxyl-radical production via Fenton/Haber-Weiss reactions. Recently, body iron reduction has been found to ameliorate diabetes. In the present study, we examined the protective effect of dietary iron restriction against diabetic nephropathy in the db/db mouse model of diabetic nephropathy using db/db mice as controls. The db/db mice were divided into two groups and fed a normal diet (ND) or a low-iron diet (LID). Increasing urinary albumin excretion was observed in the ND db/db mice, but this was suppressed in db/db mice with LID. Histologically, the db/db mice in the ND group had increased glomerular volume and mesangial area compared with the LID group. Deposited age of extracellular matrix decreased in db/db mice with LID. In terms of oxidative stress, increased superoxide production observed in the kidneys of the ND db/db mice was diminished in the LID group. NADPH oxidase activity and renal expression of NADPH oxidase components p22phox and NADPH oxidase 4 (NOX4) were augmented in the ND group, and this was abolished by LID. There were no differences in expression of renal iron importers, transferrin receptor, or divalent metal transporter-1 between db/m mice and db/db mice. The level of ferroportin, an iron exporter, increased in the kidneys of the db/db mice. Urinary iron excretion was significantly higher in ND db/db mice and was reduced in the LID group. These findings suggest that dietary iron restriction exerts a preventive effect on the progression of diabetic nephropathy partly due to the reduction of oxidative stress.

diabetic nephropathy; iron; oxidative stress; NADPH oxidase

Iron is well recognized as an elemental trace metal for essential metabolic processes in almost all living bodies. In normal conditions, intracellular iron levels are well controlled by iron transporters and iron-binding proteins, and iron is stored in various composites such as metalloproteins, heme complexes, and oxygen carrier proteins (5). On the other hand, excess iron causes oxidative stress via production of reactive oxygen species (ROS) such as hydroxyl-radicals through Fenton/Haber-Weiss reactions (28), which causes tissue damage via injuries to DNA, proteins, lipids, and carbohydrate. Physiologically, the dual nature of iron is like a double-edged sword.

Several iron overload diseases, including hereditary hemochromatosis or thalassemia, demonstrate ectopic iron accumulation with consequent complications of iron-related organ damage such as cardiomyopathy, liver cirrhosis, and diabetes mellitus caused by disturbance of pancreatic insulin secretion (6). Moreover, iron is reported to be involved in pathological conditions in patients with noniron overload disorders. For instance, hepatic iron accumulation is associated with inflammatory activity in patients with chronic hepatitis C, and iron reduction therapy by phlebotomy is a very effective treatment in these patients (21, 22). In Alzheimer’s disease, iron accumulates in senile plaques and neurofibrillary tangles (44), and the toxicity of amyloid-beta in Alzheimer’s disease is enhanced by iron (35); therefore, iron reduction therapy also has the potential to be effective for the prevention of neural damage in Alzheimer’s disease (8). Thus iron-derived oxidative stress is considered a plausible factor in the primary pathogenesis in complications of both hereditary iron overload disease and nonhereditary iron overload diseases, including hepatitis C and Alzheimer’s.

The number of patients diagnosed with metabolic syndrome has been increasing worldwide. Metabolic syndrome is a purported cause of the onset of type 2 diabetes mellitus, and it is well-known that patients with diabetes often suffer from various organ damage induced by diabetic complications, including neuropathy, retinopathy, and micro- or macroangiopathy, and diabetic nephropathy, which is a serious diabetic complication because its progression causes end-stage renal failure requiring maintenance hemodialysis or renal transplantation. Moreover, recent studies have clarified that diabetic nephropathy is closely associated with mortality or increased risk of cardiovascular events (42). Therefore, it is a crucial to elucidate the pathogenic mechanisms underlying diabetic nephropathy to develop new therapeutic approaches.

Oxidative stress plays an important role in diabetes as well as diabetic complications (19). Various mechanisms are responsible for the onset of diabetic nephropathy, and oxidative stress is also an important possible causative factor of diabetic nephropathy (31). Because iron acts as a potential catalyst of oxidative stress via the Fenton/Haber-Weiss reaction and catalytic iron is suggested to participate in the pathogenesis of diabetes and its complications (13, 45), iron reduction may potentially improve diabetes and its complications via the inhibition of iron-catalyzed oxidative stress. Some animal studies have reported that iron reduction moderates diabetes (7,
Table 1. Effects of dietary iron restriction on body weight, kidney weight, blood pressure, heart rate, blood glucose, hemoglobin, serum iron levels, kidney iron contents, and urinary iron excretion in the mice

<table>
<thead>
<tr>
<th></th>
<th>db/db Mice with ND</th>
<th>db/db Mice with LID</th>
<th>db/db Mice with ND</th>
<th>db/db Mice with LID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>30.1 ± 0.5</td>
<td>47.9 ± 1.3†</td>
<td>46.3 ± 1.0†</td>
<td>43.7 ± 1.0†</td>
</tr>
<tr>
<td>Right kidney weight, mg</td>
<td>184 ± 6</td>
<td>247 ± 5‡</td>
<td>231 ± 7‡</td>
<td>217 ± 7‡</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>116 ± 3</td>
<td>120 ± 2</td>
<td>110 ± 3</td>
<td>108 ± 3</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>601 ± 6</td>
<td>554 ± 9*</td>
<td>553 ± 9*</td>
<td>549 ± 9*</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>152 ± 8</td>
<td>689 ± 46‡</td>
<td>561 ± 37‡</td>
<td>540 ± 37‡</td>
</tr>
<tr>
<td>Fasting blood glucose, mg/dl</td>
<td>77 ± 4</td>
<td>288 ± 26†</td>
<td>231 ± 17†</td>
<td>217 ± 17†</td>
</tr>
<tr>
<td>Hemoglobin, g/dl</td>
<td>14.1 ± 0.4</td>
<td>13.5 ± 0.6</td>
<td>10.4 ± 0.4§</td>
<td>10.4 ± 0.4§</td>
</tr>
<tr>
<td>Serum iron, µg/dl</td>
<td>96 ± 6</td>
<td>215 ± 14†</td>
<td>158 ± 21†</td>
<td>145 ± 21†</td>
</tr>
<tr>
<td>Kidney iron, µg/g kidney tissue</td>
<td>7.7 ± 0.4</td>
<td>7.5 ± 0.7</td>
<td>5.7 ± 0.3‡</td>
<td>5.3 ± 0.3‡</td>
</tr>
<tr>
<td>Urinary iron excretion, µg/day</td>
<td>0.93 ± 0.122</td>
<td>2.21 ± 0.34†</td>
<td>1.18 ± 0.35‡</td>
<td>1.07 ± 0.35‡</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 11–14, respectively. ND, normal diet; LID, low-iron diet. *P < 0.05; †P < 0.122 vs. db/db mice with ND; ‡P < 0.01 vs. db/db mice with ND.

MATERIALS AND METHODS

Chemicals and reagents. The following commercially available antibodies were used for this study: anti-fibronectin antibody, anti-desmin antibody, anti-p22phox, anti-NADPH oxidase 4 (anti-NOX4) antibody, anti-ferroportin heavy and light chain antibodies, anti-NRAMP2 [divalent-metal transporter-1 (DMT1)] antibody (Santa Cruz Biotechnology, Santa Cruz, CA); anti-collagen IVa antibody (Abcam, Tokyo, Japan); anti-transferrin receptor-1 (anti-TIR-1) antibody and anti-ferroportin (anti-FPN) antibody (Alpha Diagnostics, San Antonio, TX); and anti-tubulin antibody as a loading control (Calbiochem, San Diego, CA).

Experimental animals and treatment. All animal experimental procedures were performed in accordance with the guidelines of the Animal Research Committee of the University of Tokushima Graduate School, and protocols were approved by the Tokushima University Institutional Review Board for animal protection. Male db/db mice (BKS.Cg-t+/− Leprdb/Jcl) and male db/m mice (BKS.Cg-m+−/− Leprdb/Jcl) were purchased from CLEA Japan (Tokyo, Japan). The mice were maintained in a room under conventional conditions with a regular 12-h light/dark cycle and kept with free access to food (type NMF; 360 kcal/100 g, 5.3% fat, 6.1% carbohydrates, 23.6% proteins, 2.9% fiber, 54.4% nitrogen-free extract, 7.7% fluid, and 0.01% iron (1 mg Fe/100 g food; Oriental Yeast, Tokyo, Japan)) until 8 wk of age. At 8 wk of age, db/db mice were divided into two groups; normal diet (ND)-fed group (type NMF) and low-iron diet (LID)-fed group (type NMF with Fe 0.001% (1 mg Fe/100 g food)). Mice were kept for next 8 wk, and then killed and used for analysis. Hydralazine chloride (50 mg·kg⁻¹·day⁻¹) was given to mice orally mixed in drinking water.

Blood and serum chemistry. Blood glucose levels were measured using an ACCU-CHEK Aviva kit (Roche Diagnostics, Basel, Switzerland). Serum iron levels were measured using an iron assay kit according to the manufacturer’s instructions (Metallo assay; AKJ Global Technology, Chiba, Japan).

Measurement of urinary creatinine levels and albumin excretion. The levels of urinary creatinine and albumin were measured at 8, 12, and 16 wk of age. Urinary samples were collected for 24 h using a metabolic cage. The urinary level of creatinine and albumin was determined by enzyme method and turbidimetric immunoassay, respectively (SRD, Tokyo, Japan).

Histological analysis of glomerular area and mesangial expansion. Mice were killed with intraperitoneal injection of high-dose pentobarbital. After perfusion with physiological saline, kidneys were excised. The samples were fixed overnight in 4% paraformaldehyde at 4°C. Paraffin-embedded samples were cut into 2-µm sections and stained with periodic acid-Schiff (PAS) reagent. Mesangial size was determined by the average of five different sections in each sample. To analyze glomerular area and mesangial expansion, 20 glomeruli randomly selected from each mouse were measured by PAS-positive area in the mesangial area and glomerular tuft area by ImageJ 1.38 software (National Institutes of Health, Bethesda, MD). Mesangial expansion was expressed as the mesangial positive area to glomerular tuft area ratio.

Measurement of tissue iron concentration in kidney. The iron concentration was measured by Mettalo assay kit according to the manufacturer’s instruction as described previously (25). In brief, kidney tissues were mechanically homogenized in cell lysis buffer. Uncentrifuged crude lysates with final concentration of 0.1 M HCl were well mixed every 10 min, kept at 4°C for 30 min, and then centrifuged at 4°C for 10 min. The supernatant was used for the assay. Renal iron concentration was corrected by tissue weight and expressed as micrograms per gram of tissue.

Quantitative measurement of mRNA expression levels. The procedures of total RNA extraction from kidney and cDNA synthesis are previously described (46). The methods of quantitative real-time RT-PCR were also described in our previous study (46). The primers used were as follows: 5’-TACCCACAATGCGGCTC-3’ and 5’-GCCCTAATTGGACCATGT-3’ for collagen Iα2, 5’-ACAGATCCAACTCCCTCTGA-3’ and 5’-TGCTCTCCTGGTCTTCCT-3’ for fibronectin, 5’-CAAGGGTTCTGAGTCCA-3’ and 5’-GTAGCCCTGCTGACAACCCT-3’ for desmin, 5’-TCTCATGTCGACCAAGAAGG-3’ and 5’-CTCCTAGTGTGGTCCATG-3’ for p67phox, 5’-GTCCTCGACATCTCACTGGA-3’ and 5’-ATGCCATCTGGAACCAAGAAGG-3’. The ratio of target mRNA to reference mRNA was calculated by 2-ΔΔCT method. The primer sequences and corresponding cDNA primers are listed in Supplemental Table S1. The primers of total RNA extraction from kidney and cDNA synthesis are also listed in Supplemental Table S1.
GAATGCCCTCAACT-3′ and 5′-TTCTGGGATCTCTATTCTGG-3′ for NOX4, and 5′-GCTCCAGCAGATGCA-3′ and 5′-CCGGACGTGAGGCAGCAG-3′ for 36B4 as an internal control.

Western blotting. Protein expression was examined by Western blotting. The methods of protein extraction and Western blotting were described in detail previously (46). The semiquantitative analysis of immunoblot band densitometry was performed using ImageJ 1.38 software.

Immunohistochemistry. The unfixed kidney sample was snap-frozen in liquid nitrogen and embedded in optimal cutting temperature compound for cryomolds for frozen section. Samples were cut into 7-μm sections, dried, and then fixed in 4% paraformaldehyde for 10 min before use. Paraffin-embedded kidney samples were sectioned, deparaffinized, and then processed with antigen retrieval in 10 mM citrate buffer at 95°C for 10 min before use. The tissue sections were incubated with primary antibody at 4°C overnight. Antibody distribution was visualized using immunofluorescence (Alexa fluor; Life Technology, Tokyo, Japan) or a streptavidin-biotin complex assay and a DAB substrate kit (LSAB+ Kit Universal; Dako, Tokyo, Japan). Sections incubated without primary antibody were used as negative controls. The deposition of collagen IV and fibronectin in glomeruli was quantified by fluorescence intensity × positive area (μm²) to glomerular area (μm²). The extent of the positive area of glomerular desmin was expressed as the ratio of desmin positive area to glomerular tuft area.

In situ superoxide detection. The dihydroethidium (DHE) staining method has been described previously (46). In brief, excised fat was frozen in optimal cutting temperature compound. Samples were sectioned and placed on glass slides. The sections were incubated with DHE in PBS (10 μM) in a dark, humidified container at room temperature for 30 min. After a cover glass was placed over the section, the fat tissue was observed using fluorescence microscopy.

Measurement of malondialdehyde concentration in kidney. Thiobarbituric acid reactive substances (TBARS) in the kidney were measured by using a commercially available assay kit (TBARS assay kit; Cayman Chemical, Ann Arbor, MI) as previously described (24). In brief, the kidney was homogenized and the uncentrifuged suspension was used for assay. After an equal volume of SDS solution was added, the mixture was incubated at 95°C for 60 min and then cooled to room temperature. After the sample was centrifuged, the supernatant was measured absorbance at 532 nm.

Measurement of renal NADPH oxidase activity. Renal NADPH oxidase activity was measured as previously described (46). In brief, kidney was homogenized in lysis buffer, and then sonicated for 3 s. The reaction was started by the addition of NADPH (100 μM) to a suspension containing 20–50 μg protein, lucigenin (10 μM). Luminescence was measured every 1 s for 60 min in a plate reader (SpectraMaxParadigm FilterMaxF3; Molecular Devices Japan, Tokyo, Japan).

Fig. 2. Histopathological analysis of changes in morphology of diabetic kidneys in 16-wk-old mice after 8 wk of feeding with ND or LID. Representative histological findings of periodic acid-Schiff (PAS) staining (Aa–Ac) and immunohistochemistry for collagen IV (Ad–Af), fibronectin (Ag–A1), and desmin (A2–A4). B: quantitative analysis of glomeruli size (left) and mesangial area expansion (right). Quantitative analysis for staining and mRNA expression of collagen IV (C), fibronectin (D), and desmin (E). db/m mice with ND: white bar; db/db mice with ND: black bar; and db/db mice with LID: gray bar. Data are expressed as means ± SE. *p < 0.05, **p < 0.01; n = 8 in each group.
Isolation of glomeruli from the kidney. Isolated glomerular fractions were collected from the kidneys of mice using a differential sieving method described previously (37). In brief, renal cortices were minced and pressed with an inner syringe through a 150-μm stainless steel screen mesh then rinsed with ice-cold PBS before passage through successive screen meshes of 75 and 45 μm, respectively. Glomeruli were collected from mesh screen of diameters of 45 μm, resuspended in PBS, and recollected by centrifugation. The purity of glomerular isolation was ~70% on average.

Statistical analysis. Data are expressed as means ± SE. For comparisons among the three groups, statistical significance was confirmed using a one-way ANOVA and the significance of each difference was determined by post hoc testing using the Tukey-Kramer method. P values <0.05 were considered statistically significant.

RESULTS

Characteristics of experimental mice. We first evaluated the characteristics of the three groups of mice at the end of the experimental period. As shown in Table 1, body weight and kidney weight of db/db mice were greater than those of db/m mice. Kidney weights of db/db mice in the LID group were lower than those of the db/m mice with a ND, although there were no differences of body weight between these groups. Systolic blood pressure was significantly decreased in db/db mice with LID and heart rates of db/db mice were lower than those of db/m mice. Increased levels of both fasting and casual blood glucose levels were reduced in db/db mice with LID. In regard to the iron parameter, no differences of kidney iron content were seen between db/m mice and db/db mice, whereas serum iron levels and urinary iron excretion were greater in db/db mice. LID reduced this serum iron and urinary iron excretion in db/db mice as a consequence of reduced hemoglobin levels.

Effect of iron restriction on urinary albumin excretion. By the age of 8 wk, db/db mice already had elevated urinary albumin excretion compared with db/m mice. The extent of albuminuria was greater in the ND groups at 4 and 8 wk after commencing the experiments, and this increase was suppressed among the LID db/db mice (Fig. 1).

Kidney morphology of db/db mice with LID. We examined the effect of LID on the pathohistology of diabetic nephropathy in the db/db mice. Figure 2, Aa–Ac, shows representative figures from PAS-stained kidney of the LID, ND, and control groups. Glomerular area in db/db mice with ND was larger than that of db/m mice with ND but not in LID. In the ND group, db/db mice had extensive mesangial expansion in the glomeruli compared with the db/m mice; this was suppressed in the LID db/db mice. The ratio of mesangial area to glomerular area was significantly larger in the ND db/db mice than in controls. LID feeding limited mesangial expansion (Fig. 2B).

A

B

C

D

Fig. 3. Effect of iron reduction on oxidative stress in diabetic kidneys 16-wk-old mice after 8 wk of feeding ND or LID. A, left: representative finding of dihydroethidium (DHE) staining of kidney section. A, right: relative fluorescence intensity. G, glomeruli. Values are expressed as means ± SE. n = 8 in each group. *P <0.05, **P < 0.01. B: thiorbarbituric acid reactive substances (TBARS) assay of whole kidney. Values are expressed as means ± SE. *P < 0.05, **P < 0.01; n = 6–8 in each group. C: effect of LID on renal NADPH activity. Data are expressed as means ± SE. *P < 0.05, **P < 0.01; n = 9–11 in each group. D: effect of LID on NADPH subunits in kidney. Quantitative real-time PCR analysis of mRNA expression of p22phox, NADPH oxidase 4 (NOX4), p67phox, and p47phox.

Data are expressed as means ± SE. *P < 0.05, **P < 0.01; n = 8 in each group.

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Expression of mRNA for both collagen IV and fibronectin was augmented in the kidneys of ND \(db/db\) mice compared with \(db/m\) mice with ND, but the elevations were reduced in \(db/db\) mice by LID (Fig. 2, C and D). Similarly, immunohistochemical analysis indicated that deposition of collagen IV and fibronectin in the glomeruli of \(db/db\) mice was significantly higher with ND than in controls with ND, while this effect was reduced by LID (Fig. 2, Ad–Af, Ag–Ai, C, and D). According to molecular analysis, the level of desmin, a marker of podocyte injury, increased in kidneys of \(db/db\) mice with ND and reduced in \(db/db\) mice with LID (Fig. 2, Ad–Al and E).

Effect of LID vs. oxidative stress in diabetic nephropathy. As indicated, diabetic complications such as nephropathy are associated with oxidative stress. DHE staining suggested increased renal superoxide production in \(db/db\) mice with ND vs. \(db/db\) mice with LID (Fig. 3A), and similarly, renal oxidative stress was diminished in LID when examined by TBARS assay (Fig. 3B). The levels of \(p22^{phox}\), NOX4, \(p47^{phox}\), and \(p67^{phox}\), both \(p22^{phox}\), and NOX4 mRNA expression were elevated with ND, and the effect was diminished after LID (Fig. 3D). Corresponding protein levels followed suit for \(p22^{phox}\) and NOX4 ND \(db/db\) mice, which was again ameliorated in LID \(db/db\) mice (Fig. 4, A and C). There were no differences of \(p47^{phox}\) and \(p67^{phox}\) mRNA expression in kidneys among the experimental groups (Fig. 3D). The expression of \(p22^{phox}\) and NOX4 in the proximal tubule and glomeruli and to some degree, the cortex and medulla, was higher with ND, and the effect was seen to a lesser extent with LID (Fig. 4, B and D). Renal NADPH oxidase activity was increased ND and suppressed by LID (Fig. 3C). In isolated glomeruli, NOX4 mRNA expression was higher in \(db/db\) mice with ND, and it was reduced in \(db/db\) mice with LID, while \(p22^{phox}\) mRNA expression was lower in \(db/db\) mice with both ND and LID (see Fig. 6, A and B).

Changes in expression of iron transporter, ferritin, and urinary iron excretion in diabetic kidneys. As shown Table 1, urinary iron excretion was elevated in \(db/db\) mice with ND compared with \(db/m\) mice with ND, and the effect was limited by LID. The expression of renal iron transporters such as Tfr and DMT1 did not differ between \(db/m\) mice and \(db/db\) mice with ND (Fig. 5, A and B), while renal FPN expression was increased in \(db/db\) mice with ND and restored to baseline in \(db/db\) mice with LID (Fig. 5, C and D). Tfr expression was reduced in \(db/db\) mice with LID. As shown in Fig. 5, E–G, ferritin heavy chain expression was augmented in kidneys of \(db/db\) mice with ND, and the effect was reversed in \(db/db\) mice with LID. Ferritin light chain was also decreased in \(db/db\) mice with LID, although there was no difference between \(db/m\) mice and \(db/db\) mice with ND. However, in the glomerular fraction, there were no differences between ND \(db/m\) mice and \(db/db\) mice in terms of ferritin heavy chain and ferritin light chain mRNA expression (Fig. 6, C and D). These findings suggested that NOX4 was dominant in glomeruli, while \(p22^{phox}\) mainly existed in proximal tubule. In addition, ferritin heavy chain was predominant in the proximal tubule, indicating that iron largely exists in proximal tubules.

Effect of iron restriction on diabetic nephropathy is independent of the change in blood pressure. LID significantly lowered blood pressure in \(db/db\) mice. Therefore, we checked whether the effect of iron restriction against diabetic nephropathy was dependent on the reduction in blood pressure by lowering blood pressure with hydralazine. As shown Fig. 6E, hydralazine reduced blood pressure to the same degree or even further than iron restriction but urinary albumin excretion was not suppressed compared with ND group (Fig. 6G). This finding indicated that the effect of iron restriction against diabetic nephropathy was independent of its effect on blood pressure.

**Fig. 4.** Immunoblotting and immunohistochemistry for \(p22^{phox}\) and NOX4 expression in diabetic kidney in 16-wk-old mice after 8 wk of feeding ND or LID. A, top: representative blot of \(p22^{phox}\) and tubulin. A, bottom: quantifies densitometry of \(p22^{phox}\) corrected by tubulin. Values are expressed as means ± SE. *P < 0.05; **P < 0.01; n = 8 in each group. B: representative immunohistochemical staining of \(p22^{phox}\) in cortex and medulla of kidney. C: top: representative blot of NOX4 and tubulin. B, bottom: quantifies densitometry of NOX4 corrected by tubulin. Values are expressed as means ± SE. *P < 0.05; **P < 0.01; n = 8 in each group. D: representative immunohistochemical staining of NOX4 in cortex and medulla of kidney.
We also showed that LID suppressed renal oxidative stress limitation against the development of diabetic nephropathy. Therefore, iron restriction reduces the production of oxidative stress catalyzed by the Fenton/Haber-Weiss reaction, contributing to the amelioration of diabetes and its complications.

Several studies have indicated a relationship between diabetes and body iron content. High body iron stores expressed as serum ferritin concentration have been linked to increased risk for the development of type 2 diabetes (16, 17, 26). Excess amounts of nontransferrin bound iron, the form of circulating iron that is most susceptible to redox activity, were seen in type 2 diabetic patients with a strong correlation with severity (30). On the other hand, diabetic effects were reduced by iron reduction with phlebotomy (14) or chelation therapy (9). Similar beneficial effects have been found in experimental animal studies examining dietary iron reduction (7, 39), phlebotomy (venesection; Ref. 39), and iron chelation treatment (46). Iron reduction has been shown to diminish oxidative stress in liver (39) and fatty tissues in diabetic conditions (46). A low-iron, polyphenol-enriched carbohydrate-restricted diet reduced mortality and loss of renal function in patients with diabetic nonhereditary diseases related to iron accumulation, diabetes and its complications, and experimental iron reduction reduces oxidative stress. Therefore, iron restriction reduces the production of oxidative stress catalyzed by the Fenton/Haber-Weiss reaction, contributing to the amelioration of diabetes and its complications.
nephropathy (12). Similarly, as we demonstrated, body iron content by dietary iron restriction prevented exacerbation of diabetic nephropathy and reduced renal oxidative stress, again suggesting that iron restriction is an effective method to slow the progression of diabetic nephropathy.

In the present study, iron reduction suppressed NADPH oxidase subunits p22phox and NOX4 in the kidneys of db/db mice. NADPH oxidase plays a crucial role in diabetic nephropathy (32) and apocynin, a NADPH oxidase inhibitor, prevented the progression of diabetic nephropathy (3). Similar to our results, the expression of p22phox and NOX4 were elevated in diabetic kidneys (11). In the relationship between NADPH oxidase subunits p22phox and NOX4 in the kidneys of diabetic mice, LID-fed db/db mice treated with hydralazine. Values are expressed as means ± SE. *P < 0.05, **P < 0.01 vs. ND-fed db/db mice at pretreatment of hydralazine; n = 8. F: blood pressure at 16 wk of age in ND-fed db/db mice, LID-fed db/db mice, and ND-fed db/db mice treated with hydralazine. Values are expressed as means ± SE. *P < 0.05, **P < 0.01 vs. ND-fed db/db mice at pretreatment of hydralazine; n = 8.

H: urinary albumin excretion at 16 wk of age in ND-fed db/db mice, LID-fed db/db mice, and ND-fed db/db mice treated with hydralazine. Values are expressed as means ± SE. *P < 0.05; n = 8–15 in each group.

Fig. 6. mRNA expression of p22phox (A), NOX4 (B), FTH (C), and FTL (D) in isolated glomeruli of mice at 16 wk of age. Values are expressed as means ± SE. *P < 0.05, **P < 0.01; n = 4 in each group. G: change in blood pressure in ND-fed db/db mouse with oral hydralazine (HYD). Values are expressed as means ± SE. *P < 0.01 vs. ND-fed db/db mice at pretreatment of hydralazine; n = 8. H: urinary albumin excretion corrected by urinary creatinine expressed as means ± SE. *P < 0.05, **P < 0.01 vs. ND-fed db/db mice at 16 wk of age; n = 8–14 in each group. G: urinary albumin excretion corrected by urinary creatinine expressed as means ± SE. *P < 0.05, **P < 0.01 vs. ND-fed db/db mice at pretreatment of hydralazine; n = 8.

Iron is required for enzyme activity of NADPH oxidase (41) as well as the biosynthesis of heme protein p22phox subunit (50). Therefore, iron reduction seems to contribute to the suppression of oxidative stress through the reduction of p22phox protein expression and NADPH oxidase activity. Iron is required for enzyme activity of NADPH oxidase (41) as well as the biosynthesis of heme protein p22phox subunit (50). Therefore, iron reduction seems to contribute to the suppression of oxidative stress through the reduction of p22phox protein expression and NADPH oxidase activity, leading to experimental slowing of the development of diabetic nephropathy. NOX4, a homolog of gp91phox, is highly expressed in the kidney and a source of renal oxidative stress (18, 43). Increased NOX4 expression was found in diabetic kidneys, and antisense oligonucleotides for NOX4 treatment inhibited renal oxidative stress, consequent to the reduction of renal hypertrophy and increased fibronectin expression (20). Here, we found that increased NOX4 mRNA and protein levels in db/db mice were diminished by iron restriction, contributing to the suppression of renal oxidative
stress. Moreover, Ambasta et al. (2) have shown that the interaction of NOX4 with p22phox is required to form a superoxide-generating NADPH oxidase. Taken together, the underlying mechanism of the decrease of oxidative stress by iron restriction is suggested to be involved in the NADPH oxidase pathway in diabetic kidneys in addition to the Fenton/Haber-Weiss reaction.

The kidney is probably involved in iron homeostasis (49), and changes in renal iron metabolism under the diabetic condition have been reported in several studies. In clinical studies, augmented proximal tubular lysosomal iron was observed in patients with diabetic nephropathy (40), and urinary iron excretion was increased in patients with diabetes (23). The movement of urinary proteins such as transferrin was suggested to be a source of renal and urinary iron increased due to the impairment of glomerular permeability and selectivity (1).

In an animal experiment using streptozotocin-induced diabetic rats, renal iron content was increased (29), while renal DMT1 expression was downregulated and TfR expression was upregulated (48). In the present study, urinary iron excretion and serum iron concentration were greater in db/db mice with ND and db/db mice with ND. In addition, the expression levels of FPN but not of TfR or DMT1, were increased in proximal tubule and medulla, but not the glomerular fractions, of db/db mice with ND. Although the role of renal FPN remains unclear, in phenylhydrazine-induced hemolytic anemia mice, renal FPN expression was increased in proximal tubule in cortex, but decreased in medulla, indicating prevention of urinary iron loss (47). We propose that renal iron reabsorption showed compensatory increase through increased FPN expression, and consequently, renal iron content seemed to be preserved in the diabetic kidneys of db/db mice. Moreover, we also found that renal ferritin heavy chain expression was higher in db/db mice with ND. Ferritin is an iron-binding protein for intracellular iron storage and exerts antioxidative function (3). Renal FPN expression was increased in proximal tubules and collecting ducts; therefore, this findings also supported that iron reabsorption was increased at proximal tubule in diabetic kidney in addition to augmented renal FPN expression. Elevated FTH expression might also contribute to compensatory antioxidative effects in diabetic kidney. Further investigations were necessary for clarifying the alternation and its significance of iron metabolism in diabetic nephropathy.

In conclusion, our results support dietary iron restriction as a possible means to prevent the development of diabetic nephropathy and reduce renal oxidative stress derived from both the Fenton/Haber-Weiss reaction and NADPH oxidase. This may be a new therapeutic strategy for diabetic nephropathy.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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