Iron reduction by deferoxamine leads to amelioration of adiposity via the regulation of oxidative stress and inflammation in obese and type 2 diabetes KKAY mice

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Am J Physiol Endocrinol Metab 302: E77–E86, 2012. First published September 13, 2011; doi:10.1152/ajpendo.00033.2011.—Iron is an essential trace metal for fundamental metabolic processes in living cells and organisms. In the normal state, intracellular iron levels are regulated by cellular iron transporters and iron-binding proteins, and iron is stored in various composites such as metalloproteins, heme complexes, and oxygen carrier proteins (3). However, in the presence of oxygen, excess iron, especially in the small labile iron pool, frequently leads to the formation of oxidative stress by the generation of reactive oxygen species (ROS) that include the superoxide radical anion, hydrogen peroxide, and the hydroxyl radical (21, 44). Iron-derived ROS cause organ dysfunction via damage to DNA, lipids, and proteins (1).

In addition to well-known iron-overload diseases such as hereditary hemochromatosis and thalassemia, iron accumulation has recently been shown to be involved in the pathological conditions of various other diseases. These diseases are also caused by iron overload and are complicated by cardiomyopathy, liver cirrhosis, and diabetes, which are induced by iron accumulation (4). Iron storage is associated with disease activity in patients with hepatitis C, and reduction of body iron storage by phlebotomy is effective for treatment of patients with hepatitis C (17, 18). Iron deprivation also prevents neural damage in several neurodegenerative diseases and in Alzheimer’s disease (6, 37). Additionally, some malignant tumors are attributable to accumulated iron (13). Because iron causes production of hydroxyl radicals via the Fenton/Haber-Weiss reaction (25), iron-derived oxidative stress is considered to be a main factor in the iron-associated diseases mentioned above.

Obesity is a major risk factor associated with impaired glucose tolerance and insulin insensitivity, leading to the development of type 2 diabetes mellitus (26). Recently, several studies have shown a relationship between body iron storage and obesity. Serum ferritin concentration, a useful marker of body iron storage, is linked to waist-hip ratio (14), body mass index (11), and fat accumulation (20). An elevated level of soluble transferrin receptor, another index of body iron status, has been shown to be higher and to contribute to the risk of developing diabetes in obese individuals (35).

Oxidative stress also plays an important role in the onset and progression of diabetes (36), and levels of several markers of oxidative stress are higher in obese people than in nonobese people (29, 32). Therefore, the amelioration of obesity can reduce oxidative stress and prevent the development of diabetes. Because iron is a potential catalyst of oxidative stress via the Fenton/Haber-Weiss reaction, reduction of iron levels contributes to reduced obesity via suppression of iron-catalyzed oxidative stress.

In this study, reduction of iron levels by an iron chelator ameliorated obesity through inhibition of macrophage infiltration and decreased oxidative stress and inflammation, leading to a break in a vicious cycle of adipocyte hypertrophy.

MATERIALS AND METHODS

Chemicals and reagents. Deferoxamine (DFO) was purchased from Calbiochem (Tokyo, Japan). The following commercially available antibodies were used in this study: anti-ferritin heavy chain and...
anti-p22phox (Santa Cruz Biotechnology, Santa Cruz, CA); anti-rat F4/80 antibody (AbD Serotec, Kidlington, UK); and anti-β-actin as a loading control (Cell Signaling Technology).

**Experimental animals and treatment.** All experimental procedures were performed in accordance with the guidelines of the Animal Research Committee of the University of Tokushima Graduate School. Male diabetic KKAY/Ta Jcl mice were purchased from Nippon CLEA (Tokyo, Japan). The mice were housed individually in plastic cages with free access to food (Type NMF; Oriental Yeast, Tokyo, Japan) and water throughout the experimental period.

### Table 1. Daily food intake, body weight, hematological characteristics, plasma glucose, plasma insulin, HOMA-IR, serum ferritin level, and fat iron concentration in C57BL6/J and KKAY mice at the age of 10 wk with or without DFO treatment

<table>
<thead>
<tr>
<th></th>
<th>C57BL6/J Mice</th>
<th>Vehicle-Treated KKAY Mice</th>
<th>DFO-Treated KKAY Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily food intake, g · day⁻¹ · head⁻¹</td>
<td>ND</td>
<td>6.73 ± 0.20</td>
<td>6.78 ± 0.23</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>25.8 ± 0.5**</td>
<td>41.1 ± 0.4</td>
<td>40.3 ± 0.5</td>
</tr>
<tr>
<td>Hemoglobin, g/dl</td>
<td>12.5 ± 0.4</td>
<td>12.7 ± 0.4</td>
<td>11.1 ± 0.8</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>40.9 ± 2.4</td>
<td>43.5 ± 1.6</td>
<td>40.1 ± 1.8</td>
</tr>
<tr>
<td>Fasting plasma glucose, mg/dl</td>
<td>97.6 ± 3.7*</td>
<td>118.1 ± 8.4</td>
<td>100.7 ± 4.4</td>
</tr>
<tr>
<td>Fasting plasma insulin, ng/ml</td>
<td>0.20 ± 0.04*</td>
<td>1.90 ± 0.18</td>
<td>0.57 ± 0.03*</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.08 ± 0.01**</td>
<td>0.70 ± 0.11</td>
<td>0.17 ± 0.01**</td>
</tr>
<tr>
<td>Plasma ferritin level, ng/ml</td>
<td>623.9 ± 80.0**</td>
<td>164.3 ± 6.2</td>
<td>125.6 ± 9.2**</td>
</tr>
<tr>
<td>Iron concentration in fat, µg/g tissue wt</td>
<td>20.5 ± 1.7*</td>
<td>4.9 ± 0.2</td>
<td>3.2 ± 0.4**</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; n = 4–10 mice in each group. HOMA-IR, homeostasis model assessment-insulin resistance; DFO, deferoxamine. *P < 0.05 and **P < 0.01 vs. vehicle-treated KKAY mice. ND, not done.

Fig. 1. A: effects of pharmacological iron reduction on fat weight. Weights of white adipose tissue in the epididymis, retroperitoneum, and circumintestinum in KKAY mice treated with vehicle or deferoxamine (DFO). Values are expressed as means ± SE; n = 10 mice in each group. *P < 0.05 and **P < 0.01 compared with KKAY mice with vehicle treatment. B: representative findings with hematoxylin-eosin staining of adipocytes in epididymal fat of KKAY mice with or without DFO administration. C and D: mean adipocyte size (C) and distribution of adipocyte sizes (D). Values are expressed as means ± SE, n = 6 in each group. **P < 0.01 compared with KKAY mice with vehicle treatment. E: representative findings of immunohistochemical staining for infiltrated macrophages by F4/80 antibody (black arrows) in epididymal fat of KKAY mice with vehicle (top) or DFO (bottom) administration. Panel on right shows the no. of F4/80-positive cells. Values are expressed as means ± SE, n = 5 in each group. **P < 0.01 compared with KKAY mice with vehicle treatment.
ods. The composition of the food was as follows: 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. All caged mice were maintained in a room under conventional conditions with a regular 12:12-h light-dark cycle, and temperature was maintained at 24 ± 1°C. At 8 wk of age, the KKAy mice were divided into two groups and injected intraperitoneally with either a vehicle or DFO (100 mg·kg\(^{-1}\)·day\(^{-1}\)) for 2 wk. All tissue samples were excised from overnight-fasted mice.

**Blood chemistry.** The concentrations of plasma glucose and insulin were measured, and homeostasis model assessment-insulin resistance was determined as previously described (30).

**Glucose tolerance test.** After fasting for 24 h, mice were intraperitoneally injected with 20% glucose solution (2.0 g/kg body wt). Blood was drawn from a tail vein at scheduled time points (0, 30, 60, 120, and 180 min), and blood glucose levels were measured using an ACCU-CHEK Aviva Kit (Roche Diagnostics, Basel, Switzerland).

**Insulin tolerance test.** After a 4-h fast, the mice were subjected to an insulin tolerance test. They were injected intraperitoneally with insulin (1.0 U/kg body wt; Humulin R; Eli Lilly, Indianapolis, IN). Blood samples were obtained at scheduled times (0, 15, 30, 60, and 120 min) using the ACCU-CHEK Aviva Kit.

**Measurement of serum ferritin levels.** We used a Mouse Ferritin enzyme-linked immunosorbent assay (ELISA) Kit (Immunology Consultants Laboratory, Newberg, OR) according to the manufacturer’s instructions to determine the levels of serum ferritin.

**Measurement of serum insulin levels.** We used a Mouse Insulin ELISA Kit (Morinaga Institute of Biological Science, Yokohama, Japan) according to the manufacturer’s instructions to measure serum insulin concentration.

**Measurement of urinary 8-hydroxy-2′-deoxyguanosine excretion.** The levels of serum creatinine and urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) were measured when the mice were 10 wk of age. Urinary samples were collected for 24 h using a metabolic cage (Nippon CLEA). The urinary level of creatinine was determined using an immunoassay kit (Creatinine-Test Wako; Wako Pure Chemical Industries, Osaka, Japan) according to the manu-

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**Fig. 2. Effect of iron chelation on oxidative stress in epididymal fat of KKAy mice.**

A: the effect of iron chelation on urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) excretion. Values are expressed as means ± SE, n = 6 in each group. *P < 0.05 compared with KKAy mice with vehicle treatment. B: NADPH oxidase activity in epididymal fat. Values are expressed as means ± SE, n = 6 in each group. *P < 0.05 compared with KKAy mice with vehicle treatment. C: representative images of DHE staining of epididymal fat section. Panel on right indicates relative fluorescence intensity. Values are expressed as means ± SE, n = 3 in each group. *P < 0.05 compared with KKAy mice with vehicle treatment. D: effect of DFO treatment on NADPH subunits in epididymal fat of 10-wk-old KKAy mice. Quantitative real-time PCR analysis of mRNA expression of gp91\(^{phox}\), p67\(^{phox}\), and p47\(^{phox}\). Data are expressed as means ± SE, n = 6–8 in each group. **P < 0.01 compared with KKAy mice with vehicle treatment. E: left, results of quantitative real-time PCR analysis of p22\(^{phox}\) expression; right, a representative blot of p22\(^{phox}\) and β-actin as well as the results of densitometric analysis of p22\(^{phox}\) corrected by β-actin. Values are expressed as means ± SE, n = 7–10 in each group. **P < 0.01 compared with KKAy mice with vehicle treatment. F: representative immunohistochemical staining of p22\(^{phox}\) in epididymal fat of KKAy mice with vehicle (top) or DFO (bottom) treatment. G: representative immunohistochemical staining with anti-F4/80 antibody and anti-p22\(^{phox}\) antibody in serial section in epididymal fat of KKAy mice with vehicle treatment. H: quantitative real-time RT-PCR analysis of mRNA expression of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, and monocyte chemotactic protein (MCP)-1 in epididymal fat. Data are expressed as means ± SE, n = 6–8 in each group, *P < 0.05 compared with KKAy mice with vehicle treatment.
manufacturer’s instructions. An ELISA kit (Fushimi Pharmaceutical, Kagawa, Japan) used according to the manufacturer’s instructions determined the urinary 8-OHdG concentration.

Histological analysis. Mice were killed by intraperitoneal injection of high-dose pentobarbital. White adipose tissue of the epididymal fat was removed and fixed overnight in 4% paraformaldehyde at 4°C. After defatting, the samples were cut into 10-μm-thick sections and stained with hematoxylin-eosin. Adipocyte size was determined by the average of five different sections in each sample (30).

Measurement of total iron concentration in epididymal fat tissue. The method for measurement of iron concentration has been described previously in detail (38). In brief, epididymal adipose tissue was dried at 50°C for 12 h, weighed, and transferred to an acid-washed container to which 0.5 ml of nitric acid had been added. The samples were incubated at room temperature for 1 h and then at 65°C for 4 h. After centrifugation, the supernatant was used for iron measurement. Iron concentration was corrected by tissue weight and expressed as nanogram per gram tissue.

Quantitative measurement of mRNA expression levels. RNA was extracted from epididymal white adipose tissue, and cDNA was synthesized as previously described (19). Quantitative real-time RT-PCR was performed using the iCycler MyiQ 2 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan). The primers used were as follows: 5′-CAAGATGGAGGTGGGACAGT-3′ and 5′-GCTTATCACAGCCACAGCA-3′ for gp91phox, 5′-TCTCATGCGACAGAAAG-3′ and 5′-CTCATGTGGTGGCCTTG-3′ for p67phox, 5′-GGGCTCTG-3′ and 5′-ATGACCTCAATGCTCC-3′ for p47phox, 5′-GTGGACTTTCCATTGAAGCAGCAGCT-3′ and 5′-CTCCTTACCCACTTG-3′ for p22phox, 5′-CTCGACCTGAGGAGGAG-3′ and 5′-AGGAGTTCTCAGGAAATT-3′ for tumor necrosis factor (TNF)-α, 5′-CCGAGAGGAGGAGCT-3′ and 5′-TCCGACATTTCCCAAGGAC-3′ for interleukin (IL)-6, 5′-CAGGAGGATCATTCCTGAGCAGAC-3′ and 5′-GTCGAGGAGGAGCAGATTTCTGAGCAGAC-3′ for monocyte chemotactic protein-1 (MCP-1), 5′-GGTTCGAGCAGAGCTCAGACTA-3′ and 5′-CTGGATGTGAGGCACAAGCAG-3′ for glutathione peroxidase 1 (GPx1), and 5′-GCTCCAAGCAGGAGGAGCAGCAG-3′ and 5′-CCGAGGAGGAGCAGCAG-3′ for 36B4.

Fig. 2—Continued
an internal control (19). The primer sequences for Cu/Zn-superoxide dismutase (SOD) and catalase were used as previously described (16).

**Western blotting.** The levels of protein expression were evaluated by Western blot analysis. In brief, protein extracts from samples were homogenized in T-PER reagent (Pierce Biotechnology, Rockford, IL) with a protease inhibitor cocktail and a phosphatase inhibitor (Roche Applied Science, Indianapolis, IN), as previously described (19). Densitometric analysis of immunoreactive bands was performed using Image J software (National Institutes of Health, Bethesda, MD).

**Immunohistochemical staining.** Paraffin-embedded fat tissue samples were sectioned and deparaffinized. After antigen retrieval, the tissue samples were incubated in primary antibody at 4°C overnight. Antibody distribution was visualized by a streptavidin-biotin complex assay and a DAB substrate kit (LSAB Kit Universal; Dako Japan, Tokyo, Japan). Sections incubated without primary antibody were used as negative control.

**In situ superoxide detection.** The dihydroethidium (DHE) staining method has been described previously (19). 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 mmol/l) in a dark, humidified container at room temperature for 30 min. After placing a cover glass over the section, the fat tissue was observed using fluorescence microscopy.

**Measurement of NADPH oxidase activity.** Adipose tissue was homogenized in lysis buffer (20 mmol/l KH2PO4, 1 mmol/l EGTA, and protease inhibitors; pH 7.4) and sonicated for 3 s. The homogenate was centrifuged, and the supernatant was used for determination of NADPH oxidase activity. The reaction was started by the addition of NADPH (0.1 mM) to a lucigenin-derived chemiluminescence assay was used to determine NADPH oxidase activity. Luminescence was measured every 1.8 s for 3 min in a luminometer (Varioskan Flash; Thermo Scientific, Waltham, MA). A buffer blank was subtracted from each reading. Activity was expressed as arbitrary units per milligram protein.

**Statistical analysis.** Data are expressed as means ± SE. An unpaired Student’s t-test was used for evaluation between the two groups. Statistical significance was considered at \( P < 0.05 \).

### RESULTS

**Iron status, body weight, and hematological condition after iron chelator administration.** We first investigated the effect of iron chelation on body weight and hematological findings. As shown in Table 1, pharmacological iron deprivation by DFO treatment for 2 wk diminished serum ferritin levels and fat iron concentration. Body weight tended to be decreased in DFO-treated KKAy mice (\( P = 0.06 \)), and DFO had no significant effect on hematological characteristics, including hemoglobin level and hematocrit concentration. There was no difference in daily food intake between the vehicle and DFO groups.

**Effect of DFO on adipocyte hypertrophy in adipose tissue of KKAy mice.** Next, we examined the effect of DFO on adipose tissue in KKAy mice. The weight of adipose tissue in the epididymis, retroperitoneum, and circumintestinum was diminished significantly by DFO treatment (Fig. 1A). Histological analysis showed that adipocyte size in epididymal fat was smaller in DFO-treated KKAy mice than in vehicle-treated KKAy mice (Fig. 1, B and C). The distribution of adipocyte size also indicated that DFO increased the proportion of small-sized adipocytes and decreased the proportion of large-sized adipocytes (Fig. 1D). Thus, reduction of iron level by DFO ameliorated adipocyte hypertrophy in KKAy mice. Because infiltrated macrophages are involved in adipocyte hypertrophy (41, 42), we investigated the effect of iron reduction on macrophage infiltration and accumulation in adipose tissue by immunohistochemical analysis with anti-F4/80 antibody. As
shown in Fig. 1E, the number of F4/80-positive cells was reduced significantly in DFO-treated KKAy mice. These results indicated that DFO-mediated amelioration of adipocyte hypertrophy was related to a decrease of infiltrated macrophages.

Effect of iron chelation on oxidative stress in KKAy mice. Excess iron induces production of hydroxyl radicals via the Fenton/Haber-Weiss reaction, and oxidative stress causes adipocyte hypertrophy (25). We examined the effect of iron reduction by DFO treatment on oxidative stress. DFO significantly reduced urinary 8-OHdG excretion, a marker of systemic oxidative stress (Fig. 2A). Iron reduction by DFO treatment also diminished NADPH oxidase activity and superoxide production in fat tissue (Fig. 2, B and C). Next, we investigated the expression level of NADPH oxidase, a source of ROS production, in fat. We measured the expression levels of NADPH oxidase subunits gp91phox, p67phox, p47phox, and p22phox. DFO decreased mRNA and protein expression levels of p22phox (Fig. 2E) but had no effect on mRNA expression levels of the other subunits, i.e., gp91phox, p67phox, and p47phox (Fig. 2D). We performed immunohistochemical analysis with anti-p22phox antibody to investigate the localization of p22phox in fat, which we found was largely localized in stromal tissue (Fig. 2F), and the cells expressing p22phox were also F4/80-positive macrophages (Fig. 2G). Consequently, DFO-mediated reduction of oxidative stress led to a decrease in the mRNA levels of inflammatory cytokines such as TNF-α, IL-6, IL-1β, and MCP-1 (Fig. 2H), although there were no differences in the mRNA expressions in fat of the antioxidant-related genes GPx1, Cu/Zn-SOD, and catalase between vehicle- and DFO-treated KKAy mice (Fig. 4D). These findings suggested that reduction of iron levels diminished oxidative stress through the inhibition of p22phox expression derived from macrophages.

Effect of iron chelation on iron storage protein in the fat of KKAy mice. We evaluated the location of iron in fat by tracing the iron storage protein, ferritin. Iron reduction by DFO decreased the mRNA level of the ferritin H-subunit, but not the L-subunit, in fat tissue (Fig. 4A). Immunohistochemical analysis revealed that the ferritin H-subunit was largely expressed in the stromal tissue of fat (Fig. 4B). Furthermore, ferritin H-positive cells mostly expressed F4/80 (Fig. 4C). These results suggested that iron accumulation in hypertrophied fat was mostly derived from macrophages.

Effect of DFO-induced iron reduction on oxidative stress-related gene expression in fat, skeletal muscle, and the liver. Although there was no difference in fasting blood glucose levels between vehicle- and DFO-treated KKAy mice, DFO treatment significantly reduced fasting plasma insulin levels in KKAy mice, leading to an amelioration of the hyperinsulinemic state in DFO-treated KKAy mice (Table 1). Glucose tolerance and insulin sensitivity were also improved in DFO-treated KKAy mice (Fig. 5). Consistent with our findings, it
was previously shown that animals that underwent iron chelation or dietary iron restriction exhibited amelioration of glucose metabolism compared with those that did not (5, 8, 31). Because the reduction of iron content in fat inhibited the development of adipocyte hypertrophy, it might contribute to the improvement of glucose metabolism. Additionally, we examined oxidative stress-related gene expression in skeletal muscle and the liver. Similar to our findings in fat tissue, only p22phox expression was decreased in skeletal muscle of KKAy mice with DFO administration (Fig. 3A). Expression of NADPH oxidase components in the liver was unchanged regardless of DFO treatment (Fig. 3B). The mRNA expression of

A

B

Fig. 5. Reduction of iron levels by DFO ameliorates glucose tolerance and insulin sensitivity in 10-wk-old KKAy mice. A: left, changes in blood glucose level during intraperitoneal glucose tolerance test (IPGTT). Right, area under the curve of blood glucose level during IPGTT. B: left, changes in blood glucose level during intraperitoneal insulin tolerance test (IPITT). Values are expressed as a percentage of the initial value. Right, area under the curve of blood glucose levels during IPITT. Values are expressed as means ± SE, n = 10–14. *P < 0.05 and **P < 0.01 vs. vehicle-treated KKAy mice.

Fig. 6. Effect of a low-iron diet on body weight and insulin sensitivity in KKAy mice. A: changes in body weight of 6- to 10-wk-old KKAy mice on a normal or low-iron diet. B: left, changes in blood glucose level during IPITT in KKAy mice with a normal or low-iron diet. Values are expressed as a percentage of the initial value. Right, area under the curve of blood glucose level during IPITT test. Values are expressed as means ± SE, n = 5 in each group. Low-iron diet contained 3.2 mg iron/kg diet (31).
GPx1 and Cu/Zn-SOD was also reduced in skeletal muscles but not in the liver of DFO-treated KKAy mice (Fig. 3, C and D). These results suggested that DFO-mediated iron reduction diminished oxidative stress, at least in fat and skeletal muscle, contributing to the amelioration of glucose metabolism.

**DISCUSSION**

In the present study, reduction of iron levels by DFO, an iron chelator, inhibited the development of adipocyte hypertrophy through reduction of macrophage infiltration into fat tissue, oxidative stress, and inflammatory cytokine production, leading to an improvement of glucose metabolism through improved insulin signaling in fat and skeletal muscle.

Iron storage in the body has been shown to be altered in obese people. A positive association between body mass index and serum ferritin level has been demonstrated in both men and women (11). Waist-to-hip ratio (14), a marker of central obesity, was positively correlated with serum ferritin concentration. Moreover, serum ferritin levels are also associated with the accumulation of visceral and subcutaneous fat (20). Thus, obesity is thought to be closely associated with increased iron storage in the body.

Excess iron is a potential pro-oxidant source through the production of hydroxyl radicals via the Fenton/Haber-Weiss reaction (25). Indeed, iron-catalyzed oxidative stress is thought to play a role in the pathogenesis of a variety of diseases, including diseases considered to be unrelated to iron overload (6, 13, 17, 18, 37). Oxidative stress is also thought to play an important role in the pathogenesis of obesity (22, 32). The thi et al. (39) have reported that obese people showed higher excretion of urinary catalytic iron, which is a marker of oxidative stress. Therefore, increased body iron storage is suggested to contribute to elevated oxidative stress in obese subjects.

In the present study, we demonstrated that reduction of iron level by DFO, an iron chelator, reduced oxidative stress in fat of KKAy mice. DFO reduced fat iron concentration, consequently decreasing superoxide production in fat. NADPH oxidase is a major source of superoxide production in many organs, including fat tissues (24); it plays a crucial role in the generation of ROS in obesity (12). We found that NADPH oxidase activity as well as the level of p22phox, a subunit of the NADPH component, was decreased in adipose tissue by DFO treatment. Consistent with our observation, Li and Frei reported that DFO treatment suppressed lipopolysaccharide-induced NADPH oxidase activity via attenuation of p22phox protein expression in vivo (27) and in vitro (28). Elemental iron is necessary for enzyme activity of NADPH oxidase (33) as well as the biosynthesis of heme proteins, including the p22phox subunit (43). DFO chelates iron and then blocks reaction (25). Indeed, iron-catalyzed oxidative stress is thought to play a role in the pathogenesis of a variety of diseases, including diseases considered to be unrelated to iron overload (6, 13, 17, 18, 37). Oxidative stress is also thought to play an important role in the pathogenesis of obesity (22, 32). The thi et al. (39) have reported that obese people showed higher excretion of urinary catalytic iron, which is a marker of oxidative stress. Therefore, increased body iron storage is suggested to contribute to elevated oxidative stress in obese subjects.

**Table 2. Serum ferritin level and fat iron concentration in KKAy and C57BL6/J and KK mice at the age of 4 and 10 wk**

<table>
<thead>
<tr>
<th>Age:</th>
<th></th>
<th>KKAy Mice</th>
<th>C57BL6/J Mice</th>
<th>KK Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4 wk</td>
<td>10 wk</td>
<td>4 wk</td>
</tr>
<tr>
<td>Plasma ferritin level, ng/ml</td>
<td></td>
<td>117.6 ± 11.8</td>
<td>164.3 ± 6.2**</td>
<td>397.9 ± 44.8</td>
</tr>
<tr>
<td>Iron concentration in fat, μg/g tissue wt</td>
<td></td>
<td>1.0 ± 0.5</td>
<td>4.9 ± 0.6*</td>
<td>5.3 ± 0.9</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; n = 7–10 mice in each group, *P < 0.05 and **P < 0.01 vs. age of 4-wk-old mice. ND, not done.
heme synthesis, thus affecting the cellular level of the heme protein p22phox (27). Therefore, iron level reduction can attenuate tissue oxidative stress by downregulating iron-catalyzed radical production and the activity of NADPH oxidase activity via synthesis of p22phox.

KKAy mice treated with DFO presented a smaller amount of fat tissue compared with KKAy mice receiving vehicle treatment. Macrophage infiltration into fat has been shown to be an important factor for the development of obesity. The amount of infiltrating macrophages in fat is positively correlated with adipocyte hypertrophy, and recruited macrophages are responsible as a source of inflammatory cytokines as well (41, 42). Therefore, the preventive action of DFO on development of adipose tissue hypertrophy is suggested to be exerted through the suppression of macrophage infiltration and the decrease in inflammatory cytokines in the fat of KKAy mice. Additionally, we found that p22phox expression, as well as that of the ferritin H-subunit, was colocalized with macrophages in adipose tissue of KKAy mice. Several studies have documented the association between macrophages, the p22phox subunit, and iron content. The expression of p22phox has been shown to be consistent with infiltrated macrophages in atherosclerotic plaque lesions (2) or aortic aneurysms (9). In alcoholic liver injury, hepatic macrophages have a higher iron content and show increased mRNA expression of ferritins and inflammatory cytokines such as TNF-α and macrophage inflammatory protein-1 (40). Increased numbers of macrophages with abundant iron are seen in adipose tissue in an iron-loading rat model (23, 29). Taken together with the present findings, macrophages are suggested to be a main source of iron transport and the origin of oxidative stress via NADPH oxidase in fat tissue.

Several studies have already shown favorable effects of iron reduction on diabetes. The diabetic condition in patients with type 2 diabetes was ameliorated by phlebotomy (10) and by pharmacological iron reduction (7). In experimental studies, an iron-restricted diet or phlebotomy improved the diabetic state through maintenance of pancreatic function and inhibition of oxidative stress in Otsuka Long-Evans Tokushima Fatty rats (31). Dietary iron limitation also improved glucose tolerance through preservation of β-cell function (5). Moreover, an effect of pharmacological iron reduction on the diabetic condition was shown in the pancreas of ob/ob mice (5) and in the liver of high-fat-fed rats (8). In the present study, iron reduction by DFO treatment, not a low-iron diet (Fig. 6), improved glucose tolerance and insulin sensitivity. Iron itself caused insulin resistance to glucose transport in rat adipocytes (15). In the present study, we found that DFO-induced iron reduction contributed to the decrease of oxidative stress in the fat and skeletal muscle of KKAy mice compared with vehicle treatment. Oxidative stress is well known as a key factor in the induction of insulin resistance and the development of diabetes (34). Therefore, the reduction of iron-mediated oxidative stress partly contributes to amelioration of the diabetic condition.

With DFO treatment, we demonstrated that KKAy mice exhibited reduced fat iron storage and amelioration of adipocyte hypertrophy through decreased oxidative stress and inflammation. However, the degree of participation of iron in oxidative stress and inflammation in obesity remains unclear, since various factors are involved in oxidative stress and inflammation. This is because iron storage varies between different strains of mice, regardless of whether they are lean or obese (Table 2). The results in this study were obtained using only KKAy mice and DFO. Therefore, there are limitations to our observations. Further examinations are necessary to address the issue of the involvement of iron in adiposity, for example, by employment of other animal models of obesity and other methods of iron reduction such as an iron-restricted diet or other drugs of iron chelation.

In conclusion, iron chelation diminishes macrophage infiltration, leading to reduction of oxidative stress and inflammation, which contributes to the improvement of obesity. Thus, iron level reduction might be a potential therapeutic strategy in obesity by interrupting a vicious cycle of adipocyte hypertrophy. (Fig. 7)

DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES