Iron is an essential trace metal that is involved in oxygen transport and cellular respiration. Excess iron also poses a threat to the human body because it causes oxidative stress via the Fenton/Haber-Weiss reaction. Iron homeostasis in vivo is controlled by the hepcidin/hepcidin antimicrobial peptide (Hamp)-ferroportin system (1). Iron existing in the body (mainly in senescent erythrocytes) is recycled and small losses of iron are compensated by the duodenal absorption of iron from the diet. Iron is transported into cells by divalent metal transporter-1 (DMT-1) (2). It is known that DMT-1 is regulated by transcription factor Epas1/hypoxia-inducible factor (Hif)-2α (3).

Iron overload is related to various diseases, such as cardiomyopathy (4), Alzheimer’s disease (5), and cancers (6). Ferritin is a soluble protein consisting of 24 subunits that store iron. Serum ferritin is said to correlate with total body iron stores (7). Studies have shown that the increase of serum ferritin is related to fat accumulation and is a risk factor for type 2 diabetes mellitus (8–10). Dietary iron restriction or iron chelation by the magnesium salt of (S)-3′-(OH)-DADFT polyether iron chelator was found to improve the symptoms of type 2 diabetes mellitus in ob/ob mice (11). We have previously reported that iron reduction by deferoxamine ameliorates adipocyte hypertrophy in the KKAy mouse, a model of type 2 diabetes (12). However, whether iron accumulates in the tissues with the development of diabetes is unclear.

In this study, we investigated the dynamics of iron accumulation in obese and diabetic mouse models. We generated obese and diabetic mice by feeding HFD and compared hematologic features, serum ferritin, and tissue iron contents with those of control mice. We also studied iron absorption-related gene expression in the liver and duodenum.

MATERIALS AND METHODS

Animal and tissue preparation. This study conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, 1996) and was approved by the animal care committee of the University of...
Tokushima, Japan. C57BL/6J male mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). The mice were housed in a temperature-controlled room at 25°C under a 12 h light/dark cycle and given free access to food and water. Six-week-old male mice were divided into two groups: normal diet (hereafter ND; MF, Oriental Yeast Co., Ltd., Tokyo, Japan) fed mice and high-fat diet (hereafter HFD; F2HFD2, Oriental Yeast Co., Ltd.) fed mice. ND or HFD was fed for either 3 wk or 18–20 wk.

At the endpoint of the tests, overnight-fasted animals were anesthetized by injecting sodium pentobarbital (150 mg/kg) intraperitoneally and euthanized by blood withdrawal from the inferior vena cava. The liver, duodenum, gastrocnemius muscle, heart, epididymal fat, testis, and spleen were isolated and either fixed in 4% neutral buffered formalin or snap frozen.

**Blood glucose measurement and insulin tolerance test.** Glucose levels in blood drawn from the tail vein were measured using an ACCU-CHEK Aviva Kit (Roche Diagnostics, Basel, Switzerland). Mice fed ND or HFD for 18 wk were subjected to the insulin tolerance test after a 4-h fast. Blood glucose was measured at scheduled time points (0, 15, 30, 60, 90, and 120 min) after the intraperitoneal injection of human insulin (1.0 U/kg body weight; Humulin R; Eli Lilly and Co., Indianapolis, IN).

**Blood chemistry.** Blood was collected from the inferior vena cava. Serum ferritin was measured with a Mouse Ferritin ELISA Kit (Immunology Consultants Laboratory, Inc., Newberg, OR) according to the manufacturer’s instructions. Measurements of hemoglobin, red blood cells, and hematocrit were performed by SRL, Inc. (Tokyo, Japan).

**Histological analysis.** The isolated liver and spleen were fixed with 4% neutral buffered formalin and embedded in paraffin. Sections cut into 2-μm thickness were stained with hematoxylin and eosin. The ratio of splenic red pulp area to white pulp area was determined using ImageJ 1.37V software (National Institutes of Health, Bethesda, MD).
Bethesda, MD). For iron staining, sections were cut into 3 μm thickness and stained with a Berlin blue staining set (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and nuclear fast red-aluminum sulfate solution (Merck & Co., Inc., Whitehouse Station, NJ).

**Measurement of total iron content.** Tissues were dried overnight in a vacuum chamber and their dry weights measured. The samples were dissolved in 4.5 mL of nitric acid, 2 mL of H₂O₂, and 2 mL of HCl with a MultiWave 3000 (Perkin Elmer, Inc., Waltham, MA) and diluted to appropriate iron concentrations as measured by ICP-DRC(MS)-MS (ELAN DRC II, Perkin Elmer). The operating conditions for the ICP-DRC(MS)-MS instruments were as follows: nebulizer carrier gas flow rates: Ar 1.01 L/min, NH₃ 0.6 mL/min; dwell time 50 ms/channel; data point mode-peak hopping mode; number of scans 40; repeat count 3. Values were normalized to tissue dry weight.

**Quantitative real-time RT-PCR analysis.** Tissues were homogenized and total RNA was extracted with TRIzol Reagent (Life Technologies, Inc., Rockville, MD). One microgram of total RNA was used for cDNA synthesis. Reverse transcription was performed using a QuantiTect Reverse Transcription Kit (QIAGEN, Valencia, CA). Quantitative PCR amplification was carried out with an iCycler MyiQ2 Real-Time PCR Detection System (BIO-RAD, Hercules, CA) and a Thunderbird SYBR qPCR mix (Toyobo, Osaka, Japan). The following gene-specific primer pairs were used for PCR amplification: Hamp, 5′-CTGCCTGTCTCCTGCTTCTC-3′, 5′-AGATGCAGATGGGAAAGTTC-3′; Ferroportin-1, 5′-CCCTGCTCTGGCTGTAAAAG-3′, 5′-GGTGGGCTCTTGTTCACATT-3′; DMT-1 (with iron-responsive element: IRE), 5′-GAGTAGCGGCAGCTTGTCTT-3′, 5′-ATCACAGCACGTTCACAA-GC-3′; DMT-1 (non-IRE), 5′-CTCAGGTCTTCCTGGA-CAGC-3′, 5′-CGCGTAGAGTGGGAAGAAAG-3′; Epas1/Hif2Α, 5′-CTAAGTGGCCTGTGGGTGAT-3′, 5′-CGAAGTCCTTTGCAAACCTC-3′; F4/80, 5′-CTGTAACCGGATGGCACAAT-3′, 5′-CGAGCAGATGGGAAAGTTC-3′; and 36B4 (as the internal control), 5′-GCTCCAAGCAGATGCAGCA3′, 5′-CCGGATGTGAGGCAGCA3′.

**Table 1. Iron intake and excretion.**

<table>
<thead>
<tr>
<th></th>
<th>ND (17–18 wk)</th>
<th>HFD (17–18 wk)</th>
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<tbody>
<tr>
<td>Food intake (g/d)</td>
<td>4.15±0.53</td>
<td>2.42±0.13</td>
</tr>
<tr>
<td>Dietary iron (μg/g)</td>
<td>216.75±34.25</td>
<td>141.4±3.88</td>
</tr>
<tr>
<td>Stool dry weight (g/d)</td>
<td>0.93±0.14</td>
<td>0.26±0.09</td>
</tr>
<tr>
<td>Iron per dry weight of stool (μg/g)</td>
<td>1.052.58±27.86</td>
<td>1.350.47±167.25</td>
</tr>
<tr>
<td>Stool iron per g diet (μg/g)</td>
<td>234.77±10.87</td>
<td>146.36±41.58</td>
</tr>
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</table>

Dietary iron and stool iron were measured by ICP-DRC(MS)-MS as described in “Materials and Methods.” Values are expressed as means±SD, n=3 (dietary iron in ND), n=4 (dietary iron in HFD), n=5 (food intake, stool dry weight, and amount of iron per dry weight of stool). Amount of stool excreted per day was divided by the amount of food consumed and stool iron per g diet was calculated.

**Fig. 2.** Hematologic features and serum ferritin levels of HFD-fed mice. (A–C) Blood was collected via the inferior vena cava. Hemoglobin (A), hematocrit (B), and red blood cell (C) counts were measured by SRL, Inc. Values are expressed as means±SD, n=3 in each group, *p<0.05, **p<0.01. (D) Serum ferritin levels were measured by ELISA. Values are expressed as means±SD, n=7 in each group, *p<0.05.
Statistical analysis. Data are presented as means ± SD. One outlier was rejected by Smirnov-Grubbs’ test. The unpaired Student’s two-tailed t-test was used to compare the difference between two groups. Two-way analysis of variance (ANOVA) (feed period and diet type) was used to compare the difference among four groups. As a post-hoc test, multiple comparisons with the Tukey-Kramer method were done when an interaction was observed. Differences between groups were considered significant at $p < 0.05$.

RESULTS

Generation of HFD-fed mice

Obese and diabetic mice were induced by feeding HFD for 18 to 20 wk. The amount of iron intake was low in HFD-fed mice compared to ND-fed mice (control) (Fig. 1A). Daily iron intake was constant throughout the feeding period (data not shown). Both HFD and ND were considered to contain a sufficient amount of iron because the amount of iron in the stool was almost the same as the amount of iron present in the diet (Table 1). Body weight gradually increased (Fig. 1B) and the fasting glucose level significantly increased in mice fed HFD for 20 wk (Fig. 1C). Insulin tolerance was noted in mice fed HFD for 18 wk (Fig. 1D). The HFD-fed mice had fatty livers (Fig. 1E).

Hematologic features and serum ferritin levels of HFD-fed mice

Iron in tissues of HFD-fed obese and diabetic mice

To investigate iron distribution in the body, iron con-
The iron contents in various tissues were measured by ICP-DRC(MS)-MS. Iron contents in gastrocnemius muscle, heart, epidymal fat, and testis did not change in the HFD-fed mice (Fig. 3A–D). In addition, iron contents in the liver and duodenum per dry weight were reduced to approximately half in the HFD-fed mice (Fig. 3E and G). Total liver weight of the HFD-fed mice was about two times greater than that of the ND-fed mice because of fatty liver (Figs. 1E and 3F). On the other hand, spleen iron content was markedly increased in the HFD-fed mice (Fig. 3H). In summary, iron accumulation in the HFD-fed diabetic mice was tissue-specific; the accumulation occurred specifically in the spleen.

**Morphological changes in spleen**

As iron was found to accumulate specifically in the spleen, we examined the morphology of the spleen. The spleen was large in the HFD-fed mice (Fig. 4A). In particular, the percentage of splenic red pulp showed an increase in the HFD-fed mice (Fig. 4B and C). Many cavities were noted in the splenic sinus of the HFD-fed mice (Fig. 4D). Iron accumulated mainly in the splenic red pulp (Fig. 4E). Moreover, the mRNA expression of macrophage markers F4/80, CD68, and CD11b increased in the HFD-fed mouse spleen (Fig. 4F–H).

**Changes in iron absorption related gene expression in duodenum of HFD-fed mice**

Finally, we examined the expression of iron absorption-related genes in the HFD-fed obese and diabetic mice. As fasting glucose levels did not change in the HFD-fed mice for 3 wk (Fig. 1C), we used short-term...
Iron Distribution in High-Fat Diet-Fed Mice

(3-wk)-fed mice as a dietary control. There was no difference in the expression of hepatic Hamp, the gene that encodes the liver-secreted iron-regulatory peptide hepcidin, between the HFD-fed mice and the ND-fed mice (Fig. 5A). Correspondingly, expression of ferroportin-1 in the duodenum, which is regulated by hepcidin, did not change between the HFD-fed mice and the ND-fed mice (Fig. 5B). On the other hand, the expression of the iron transporter DMT-1 (with an iron-responsive element) was elevated in the duodenum of HFD-fed mice (fed a high-fat diet for 18–20 wk) (Fig. 5C). The expression of the non-iron-responsive element DMT-1 was higher in mice fed with HFD regardless of the duration of the feeding period (Fig. 5D). Additionally, HFD-fed mice showed increased expression of duodenal Epsa1/Hif2α mRNA (Fig. 5E). Therefore, the levels of DMT-1 and Epsa1/Hif2α in the duodenum of HFD-fed mice were increased without the regulation of hepcidin and ferroportin.

**DISCUSSION**

The blood hemoglobin level, serum ferritin level, and splenic iron content were increased in the HFD-fed mice, whereas iron content in the duodenum was decreased. The relationship between the regulator of iron metabolism, hepcidin/Hamp, and type 2 diabetes remains controversial (13, 14). The expression of hepcidin/Hamp reflects iron storage in the liver (15). We found no difference in hepatic hepcidin/Hamp mRNA expression between the HFD-fed mice and the ND mice. The expression of hepcidin, iron absorption-related genes, and iron absorption is closely related, and DMT-1 expression is induced by iron deficiency in control of hepcidin/Hamp regulation (16). However, our results showed that DMT-1 expression was enhanced in the duodenum without hepcidin/Hamp regulation (16). Here we showed up-regulated Epsa1/Hif2α expression in HFD-fed mice.

Iron content in intestinal crypt cells is an important determinant of absorption (18). Increased iron absorption, a condition characterizing hemochromatosis, is
consistent with the iron status of enterocytes, but the iron status of enterocytes is inappropriately low for the body iron status in hemochromatosis (18). In addition, DMT-1 expression is inversely regulated by cellular iron concentration (18). In our model, iron concentration is low in the duodenum of HFD-fed mice. The low concentration of iron in the duodenum may induce the upregulation of DMT-1 in the duodenum of HFD-fed diabetic mice.

Iron exists mainly in red blood cells, liver, spleen, and muscle. In hemochromatotic patients, iron accumulates in the liver and heart, causing serious damage to those organs. Besides, chronic hepatitis patients present with hepatic iron accumulation and severe liver damage. In obese and diabetic mice induced with HFD for 18–20 wk, iron accumulated not in the liver and heart, but in red blood cells and the spleen. The results suggest that the distribution of iron in the body varies with the disease type.

One of the main functions of the spleen is blood filtration. Deformed or senescent red blood cells that can no longer pass through the sinus of the spleen wall because of decreased cell membrane elasticity are processed by macrophages at the splenic red pulp. The splenic red pulp is largely composed of cordal macrophages that are interconnected by their cytoplasmic processes (19). The cordal macrophages form a reticular meshwork that provides structural support to venous sinuses (19). Aged erythrocytes are phagocytized by spleen macrophages, and iron derived from them is recycled or stored in macrophages in the spleen (20). It is likely that iron accumulates in spleen macrophages of HFD-fed mice because increased percentages of splenic red pulp and macrophages were observed.

Reports of the relationship between the spleen and lipid metabolism have emerged: splenectomized rodents showed high cholesterol levels (21), hyperlipidemia (22), and increased intrahepatic fat accumulation (23). Elucidating the kinetics of iron in diabetes and other diseases is expected to lay the foundation for novel iron-targeting therapies in the future.

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