Iron restriction improves type 2 diabetes mellitus in Otsuka Long-Evans Tokushima fatty rats

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1Department of Gastroenterology/Biological Safety Science, Kyoto Prefectural University of Medicine, Kyoto; 2Department of Second Surgery, Graduate School of Medicine, Osaka City University, Osaka; 3Department of Medical Life Systems, Faculty of Life and Medical Sciences, Doshisha University, Kyoto; and 4Department of Anti-Aging food Science, Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama University, Okayama, Japan

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Iron restriction improves type 2 diabetes mellitus in Otsuka Long-Evans Tokushima fatty rats. Am J Physiol Endocrinol Metab 298: E1140–E1149, 2010. First published March 9, 2010; doi:10.1152/ajpendo.00620.2009.—Accumulating evidence suggests that alcohol, hepatitis C virus infection, steatosis with obesity, and insulin resistance are accompanied by iron overload states. Phlebotomy and oral iron chelators are effective treatments for these conditions and for hemochromatosis. However, the mechanisms by which iron depletion improves clinical factors remain unclear. We examined the effect of iron depletion in a model of type 2 diabetes, Otsuka Long-Evans Tokushima Fatty (OLETF) rats. Age-matched Long-Evans Tokushima Otsuka (LETO) rats were used as controls for all experiments. Iron restriction was performed by eliminating iron in the diet from 15 wk of age or by phlebotomy. Phlebotomy was commenced at 29 wk of age by removing 4 and 3 ml of blood from the tail vein every week in OLETF and LETO rats, respectively. Rats were euthanized at 43 wk of age, and detailed analyses were performed. The plasma ferritin concentration was markedly higher in OLETF rats and decreased in iron-deficient (ID) diet and phlebotomy rats. Hemoglobin A1c (Hb A1c) was decreased significantly in OLETF rats fed the ID diet and in the phlebotomy group. Increased levels of triglycerides, glucose, free fatty acids, and total cholesterol were found in ID OLETF rats. Plasma, liver, and pancreas lipid peroxidation and hepatic superoxide production decreased in both groups. Pancreatic fibrosis and insulin levels improved significantly in both groups of OLETF rats. Pancreatic levels of peroxisome proliferator-activated receptor-β (PPARβ) ligands and hypoxia-inducible factor (HIF)-1α were decreased significantly in OLETF rats. These factors were normalized in both rats fed ID and phlebotomy groups of OLETF rats. In conclusion, iron depletion improved diabetic complications by inhibition of oxidative stress and TGFβ signal pathways and the maintenance of pancreatic PPARβ/δ and HIF-1α pathways.

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THE INCREASE OF TOTAL BODY IRON STORES was reported to be associated with an increased risk of the development of type 2 diabetes mellitus (7–9, 11). Transferrin and iron induce insulin resistance in terms of altered glucose transport in adipocytes through a mechanism independent of fatty acids (10). Hereditary hemochromatosis frequently accompanies diabetes, and iron reduction by phlebotomy (PH) is the treatment of choice (20, 21). However, diabetes does not represent a state of iron overload that is as severe as that in hemochromatosis. Thus, whether type 2 diabetes can be improved by aggressive iron restriction is not yet known. Therefore, this study was performed to evaluate the effects of iron restriction in type 2 diabetic Otsuka Long-Evans Tokushima fatty (OLETF) rats.

It has been suggested that iron accumulation contributes to increased free radical formation and oxidative stress. The generation of reactive oxygen species (ROS) by metal oxidants such as iron may be involved in the cell damage that occurs in some diseases, such as steatohepatitis and Alzheimer’s disease (3, 30, 31). Furthermore, altered iron homeostasis has been reported in rat models of diabetes (7, 23).

Peroxisome proliferator-activated receptors (PPARs) exert important lipid-lowering effects in vivo. PPARs oppose the development of lipid-induced insulin resistance by relieving the inhibition of insulin-stimulated glucose disposal in muscle and lowering the threshold for augmented glucose-stimulated insulin secretion to counter lipid-induced insulin resistance (33). PPARβ/δ ligands were shown to be effective for the treatment of diseases such as metabolic syndrome, diabetes, and obesity (18). Recent studies have revealed a potential role of PPARβ in the regulation of glucose metabolism and insulin sensitivity (18). These actions could explain the apparently beneficial effects of synthetic PPAR agonists on circulating lipids, insulin resistance, and obesity that have been reported in some animal models (15, 24). Activation of PPARβ protected the heart from ischemia-reperfusion injury in Zucker fatty rats, and this cardioprotection seems to involve multiple mechanisms, including amelioration of lipotoxicity anti-inflammatory and upregulation of prosurvival signaling (36). PPARβ is the predominant PPAR isoform in islets (2), but its role in the pancreas is largely unknown. Furthermore, it was recently reported that iron depletion by deferoxamine upregulates glucose uptake and increases insulin receptor activity and signaling in hepatocytes in vitro and in vivo (6). In this study, we examined whether iron restriction affects PPARβ and other related molecules in a model of type 2 diabetes.

MATERIALS AND METHODS

Animals. Male OLETF rats (aged 4 wk) and age-matched Long-Evans Tokushima Otsuka (LETO) rats were obtained from the Animal Center of Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan) and were maintained until they reached an appropriate age for experiments. Rats had free access to standard laboratory chow (MF; Oriental Yeast, Tokyo, Japan) and tap water and were cared for in accordance with the specifications outlined in the National
Institutes of Health’s Guiding Principles for the Care and Use of Laboratory Animals and with approval of the Authorities of the Local Committee on Experimental Animal Research, Graduate School of Medicine, Osaka City University. LETO (n = 16) and OLETF (n = 16) rats were fed control chow and divided into five groups at 15 wk of age after the body weights of all rats were checked: ad libitum-fed controls (LETO, n = 6; OLETF, n = 6), rats fed an iron-deficient (ID) diet (LETO-ID, n = 5; OLETF-ID, n = 5), and OLETF rats that underwent regular PH (LETO-PH, n = 5; OLETF-PH, n = 5). The ID diet (Oriental Yeast) contained 0.32 mg iron/100 g diet vs. 10.9 mg iron/100 g in the control diet. The LETO-PH and OLETF-PH groups were established by withdrawing 3 and 4 ml of blood from the tail vein under diethyl ether anesthesia every week between 29 and 42 wk of age in LETO and OLETF rats, respectively. Blood was collected via a tail vein with a heparinized syringe through a 26G butterfly needle, and the same volume of saline was infused via the same route.

Animals were euthanized at 43 wk of age under urethane anesthesia (5 g/kg ip). Blood was collected with heparinized syringes, and some tissues were dissected out and frozen in liquid nitrogen. Plasma samples were obtained by centrifugation at 12,000 g for 5 min. Small pieces of liver and pancreas were removed for morphometric, immunohistochemical, or biochemical analysis.

Fig. 1. Iron staining of liver and pancreas. Animals were euthanized at 43 wk of age, as described in MATERIALS AND METHODS. Rats were made iron deficient by withholding iron beginning at 15 wk of age. In phlebotomy groups, 4 ml of blood was drawn from the tail vein every week after 29 wk until euthanasia. Sections were stained by Perls’ Prussian blue. Iron deposits of the liver (A–D) and the pancreas (E–H) can be visualized as blue. A and E: Long-Evans Tokushima Otsuka (LETO) control rats. B and F: Otsuka Long-Evans Tokushima Fatty (OLETF) control rats. C and G: OLETF-ID. D and H: OLETF-PH. Scale bars, 40 (liver) and 80 μm (pancreas).
Table 1. Changes in body weight, HCT, and Hb

<table>
<thead>
<tr>
<th>Age, wk</th>
<th>LETO</th>
<th>ID</th>
<th>PH</th>
<th>OLETF</th>
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<tbody>
<tr>
<td>29</td>
<td>489.7 ± 11.7</td>
<td>471.7 ± 9.2</td>
<td>480.8 ± 14.5</td>
<td>636.7 ± 17.3††</td>
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<tr>
<td>43</td>
<td>524.0 ± 8.8</td>
<td>478.5 ± 8.2†</td>
<td>564.4 ± 22.0</td>
<td>625.2 ± 36.5</td>
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<td>29</td>
<td>42.0 ± 0.4</td>
<td>37.0 ± 0.7†</td>
<td>42.0 ± 0.5</td>
<td>42.7 ± 0.8</td>
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<tr>
<td>43</td>
<td>55.6 ± 1.9</td>
<td>37.6 ± 1.6**</td>
<td>51.2 ± 0.8</td>
<td>52.5 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>14.1 ± 0.1</td>
<td>12.4 ± 0.2**</td>
<td>14.2 ± 0.1</td>
<td>15.3 ± 0.3</td>
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<tr>
<td>29</td>
<td>17.9 ± 0.5</td>
<td>11.9 ± 0.3**</td>
<td>16.6 ± 0.3</td>
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<tr>
<td>43</td>
<td>17.1 ± 0.3</td>
<td>11.6 ± 0.4**</td>
<td></td>
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</table>

Values are means ± SE (n = 5–6). HCT, hematocrit; LETO, Long-Evans Tokushima Otsuka rats; ID, iron deficient; OLETF, Otsuka Long-Evans Tokushima fatty rats; PH, phlebotomy. ††P < 0.01 vs. the control LETO group; *P < 0.05 and **P < 0.01 vs. each control group.

Biochemical measurements. Hematocrit and hemoglobin were measured with an automated hematology analyzer (Celluc a, MEK-6258; Nihon Kohden, Tokyo, Japan). Plasma ferritin was determined using an ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan). Plasma interleukin-6 (IL-6) was determined by a rat insulin ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan). Plasma insulin levels were determined using a supersensitive test meter (Glutest Ace; Sanwa Kagaku Kenkyusho, Tokyo, Japan) and were separated on 15% SDS-polyacrylamide gels and transferred to PVDF membranes (Immobilon-PSQ; Millipore, Billerica, MA). Determination of intracellular superoxide generation. Hepatic superoxide production was determined as described previously (22). Briefly, the oxidative fluorescent dye dihydroethidium (DHE; Wako Chemicals, Osaka, Japan) was used to evaluate the in situ production of superoxides. Unfixed fresh aortic wall and heart tissue were frozen in optimum cutting temperature compound and cut into 10-μm-thick sections and placed on glass slides. DHE (40 μM) was applied topically to each tissue section, and the slides were incubated in a light-protected, humidified chamber at 37°C for 30 min, as described previously. Images were obtained with a confocal laser-scanning microscope (LSM-510; Carl Zeiss) equipped with a krypton-argon laser. The laser settings were identical for the acquisition of images from all specimens. Fluorescence was detected with a 585-nm long-pass filter. Some specimens incubated with SOD (100 U/ml) exhibited no intensity. The intensity was semiquantified by image analyzer software (Image J). Neither signal exhibited significant photo bleaching during the time frame required for analysis. The findings reported here were replicated in three independent experiments.

Histology and immunohistochemistry. Liver and pancreas tissue samples were fixed immediately in 10% buffered formalin. All tissues were embedded in paraffin within 48 h of formalin fixation and cut to a thickness of 4 μm immediately before staining. Histological staining was performed using hematxylin-eosin or Perls’ Prussian blue method to direct iron deposits (32) and insulin. Immunohistochemistry was performed using rabbit streptavidin-biotin peroxidase kits (Vectastain Universal Elite ABC Kit; Vector...
Table 2. Plasma TG, glucose, FFA, T-chol, HDL, LDL, and IL-6 levels

<table>
<thead>
<tr>
<th></th>
<th>LETO</th>
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<th>PH</th>
<th></th>
<th>OLETF</th>
<th></th>
<th>PH</th>
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<tr>
<td>TG, mg/dl</td>
<td>40.6 ± 11.0</td>
<td>25.8 ± 3.1</td>
<td>44.8 ± 2.7</td>
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<td>334.3 ± 56.1††</td>
<td>143.0 ± 34.6**</td>
<td>457.7 ± 73.2</td>
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<td>Glucose, g/dl</td>
<td>167.4 ± 27.7</td>
<td>303.0 ± 42.5</td>
<td>241.5 ± 19.0</td>
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<td>435.5 ± 34.7††</td>
<td>343.7 ± 14.8*</td>
<td>384.3 ± 40.0</td>
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<td>FFA, µEq/l</td>
<td>712.6 ± 59.2</td>
<td>320.0 ± 32.1**</td>
<td>527.0 ± 39.7*</td>
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<td>631.7 ± 62.9</td>
<td>364.7 ± 10.8**</td>
<td>933.3 ± 139.9</td>
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<td>T-chol, mg/dl</td>
<td>111.8 ± 5.1</td>
<td>70.0 ± 2.3**</td>
<td>102.3 ± 1.7</td>
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<td>170.5 ± 6.7††</td>
<td>97.3 ± 6.5**</td>
<td>155.3 ± 16.0</td>
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<tr>
<td>LDL, mg/dl</td>
<td>24.2 ± 1.2</td>
<td>16.8 ± 2.5**</td>
<td>23.6 ± 1.0</td>
<td></td>
<td>42.8 ± 3.4†</td>
<td>24.3 ± 1.8**</td>
<td>39.8 ± 2.2</td>
</tr>
<tr>
<td>FFA, mg/dl</td>
<td>21.8 ± 0.6</td>
<td>9.8 ± 0.9*</td>
<td>15.6 ± 1.7</td>
<td></td>
<td>29.3 ± 1.0†</td>
<td>15.3 ± 0.7**</td>
<td>25.5 ± 1.6</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>106 ± 3</td>
<td>109 ± 2</td>
<td>ND</td>
<td></td>
<td>123 ± 3†</td>
<td>104 ± 4*</td>
<td>107 ± 3*</td>
</tr>
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</table>

Values are means ± SE (n = 5–6). TG, triglyceride; FFA, free fatty acids; T-chol, total cholesterol; ND, no data. TG, glucose, FFA, T-chol, HDL, and LDL were measured at 43 wk of age. †P < 0.05 and ††P < 0.01 vs. the control LETO group; *P < 0.05 and **P < 0.01 vs. each control group.

Laboratories, Burlingame, CA). Antibodies for immunohistochemical staining were directed at insulin (Dako, Tokyo, Japan). Negative control sections were processed identically, except that the primary antibody was replaced with normal mouse immunoglobulin 1.

RNA isolation, cDNA synthesis, and real-time quantitative PCR assay. Total cellular RNA was extracted from the tissues according to the manufacturer’s protocol (Qiagen, Hilden, Germany). RNA was extracted using the RNaseq midi kit from Qiagen (Valencia, CA) in accordance with the manufacturer’s instructions. The cDNA preparation was performed using 1 µg RNA in a 20-µl reaction volume according to the instructions of the ReverTra Ace quantitative (q)RT-PCR kit (Toyobo, Osaka, Japan). The reaction was carried out for 1 h at 45°C, followed by reverse transcriptase inactivation for 5 min at 95°C. The gene expressions of PPARα, PPARδ, and PPARγ were determined by real-time qRT-PCR assays.

Quantification of mRNA from the above-mentioned genes was achieved using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). RT-PCR was based on the TaqMan fluorogenic detection system (TaqMan; PE Applied Biosystems), using a fluorogenic oligonucleotide probe designed to hybridize to the specific target sequence. The TaqMan probes were labeled at the 5’ end with the fluorescent reporter dye FAM (6-carboxyfluorescein) (R) and at the 3’ end with the quencher dye TAMRA (6-carboxytetramethylrhodamine) (Q). The sequences of the gene-specific forward and reverse primers and the probes were designed using Primer Express 1.0 software (PE Applied Biosystems). The following primers and probe were used for RT-PCR of PPARα mRNA: 5’-ACTATGGAGGTCACGCCATGTTG-3’ (forward), 5’-TTTCTGTGACCAGCAGTTTACGC-3’ (reverse), and 5’-GAAGGCTGAAGGCTTTCTCCTGCGG-3’ (probe) (GeneBank, accession no. NM_013196); PPARδ mRNA: 5’-TCACCTG-GCAAGTCCACCCAGCA-3’ (forward), 5’-ACACCAGGGCCTTCTCCTGCT-3’ (reverse), and 5’-GAACGCACCCCTTCATCATCCAGA-3’ (probe).

Fig. 3. Lipid peroxide levels in plasma, liver, and pancreas. Animals were treated as described in MATERIALS AND METHODS. Levels of plasma, liver, and pancreas lipid peroxidation were measured at 43 wk. Values are means ± SE (n = 5–6). †P < 0.05 and ††P < 0.01 vs. LETO Cont; *P < 0.05 and **P < 0.01 vs. each Cont group.

Fig. 4. Plasma superoxide-dismutase (SOD)-like activity. Animals were treated as described in MATERIALS AND METHODS. Plasma SOD-like activity was determined by SOD-WST kit. Values are means ± SE (n = 5–6). †P < 0.05 vs. LETO control; **P < 0.01 vs. each control group.
(GeneBank, accession no. NM_013141); and TGFβ1 mRNA: 5'-TGCTTC-CGCATCACCGT-3' (forward), 5'-TAGTAGACGATGGGCAGTGCC-3' (reverse), and 5'-GCTGCGTGCCAGGTCTTTTG-3' (probe) (GenBank, accession no. X52498). PPARγ (Rn0040945_m1) was measured by a commercially available TaqMan Gene Expression Assay Kit (P/N 4335626; PE Applied Biosystems).

Quantification of expression of housekeeping genes. The standard curve method was used to quantify the results obtained by RT-PCR for 18S. A commercially available standard of 18S (4319413E; PE Applied Biosystems) was amplified at five different DNA template concentrations: 6.25, 12.5, 25.0, 50.0, and 100.0 ng/25 μl. Values of copy numbers for the standards were calculated on the basis of the relationship that 1 ng of DNA is equal to 333 genome equivalents (TaqMan PCR Reagent Kit Protocol P/N 402823). Amplification plots for each dilution of control template were used to determine the threshold cycle (CT) value. A standard curve was generated by plotting the CT values against the log of the known input DNA copy numbers.

Quantification of expression of target genes. To determine the quantity of the target gene-specific transcripts present in treated cells relative to untreated ones, their respective CT values were first normalized by subtracting the CT value obtained from the 18S control

Fig. 5. Superoxide generation in the liver. Frozen liver sections were prepared as described in MATERIALS AND METHODS. Fluorescent photomicrographs of confocal microscopic sections of liver samples were labeled with the oxidative dye dihydroethidium, which emits a red fluorescence when oxidized to EtBr by superoxide. Top: control rat livers in LETO (left) and OLETF (right). Middle: ID-treated livers in LETO (left) and OLETF (right). Bottom right: a representative image of a phlebotomy (PH)-treated liver of OLETF rat section. Bottom left: the figure showed a number of pixels of red area intensity semi-quantified by Image J corresponding to each photomicrograph.
The concentration of gene-specific mRNA in treated samples relative to untreated samples was calculated by subtracting the normalized C\(_T\) values obtained for untreated samples from those obtained for treated samples (\(\Delta C\_T = C\_T\_treated - C\_T\_control\)), and the relative concentration was determined (2^{-\Delta C\_T}).

Statistical analysis. In the analysis of control gene expression, all results are expressed as means ± SE, with each experiment performed in triplicate. Unless stated otherwise, results are presented as means ± SE. Results were considered significant at \(P < 0.05\). Statistical analysis was performed by analysis of variance.

RESULTS

Iron deposition in liver and pancreas. Figure 1 shows a representative iron staining section of the liver and pancreas. In LETO rats, there were no iron deposits in the liver (Fig. 1A) or pancreas (Fig. 1B). In OLETF control rats, many iron deposits were observed in the liver (Fig. 1B) and pancreas (Fig. 1F). Positive spots (blue color) were found in hepatocytes and Kupffer cells around the central vein in the liver and macrophages in the pancreas. Sections from ID and PH OLETF rats revealed the absence of iron deposits in the liver (Fig. 1, C and D) and pancreas (Fig. 1, G and H).

Changes in body weight, hematocrit, and hemoglobin. OLETF rats were significantly heavier than LETO rats (\(P < 0.01\); Table 1). Iron depletion did not affect the body weight in any group of LETO or OLETF rats. Hematocrit and hemoglobin levels were similar in LETO and OLETF rats but lower in the ID groups. The levels were slightly lower in the PH group than in the control rats.

Plasma ferritin levels. Plasma ferritin levels were decreased significantly in the ID and PH groups of OLETF rats compared with changes in LETO rats (Fig. 2).

Changes in Hb A\(_1c\), and other plasma biochemical parameters. Hb A\(_1c\) continued to increase in an age-dependent manner in the OLETF control rats (Supplemental Fig. S1; Supplemental Material for this article can be found on the AJP-Endocrinology and Metabolism web site). ID and PH returned Hb A\(_1c\) to normal levels in the OLETF rats. Table 2 shows the plasma levels of TG, glucose, FFA, total cholesterol, HDL, LDL, and IL-6 at 43 wk. All parameters, except FFA, were significantly greater in control OLETF rats than in age-matched LETO rats. The ID diet decreased FFA (45% of control in OLETF-ID and 45% of control in LETO-ID), total cholesterol (57% of control in OLETF-ID and 63% of control in LETO-ID), HDL (70% of control in OLETF-ID and 70% of control in LETO-ID), and LDL (45% of control in OLETF-ID and 45% of control in LETO-ID).

In addition, the OLETF-ID group had significantly lower TG and glucose levels. PH did not affect these parameters in either LETO or OLETF rats. ID and PH significantly decreased the plasma IL-6 levels in OLETF rats.

Oxidative stress. Plasma, liver, and pancreas lipid peroxide levels are shown in Fig. 3. The plasma, liver, and pancreas lipid peroxide levels were significantly higher in control OLETF rats than in control LETO rats. The ID diet in both groups of rats

<table>
<thead>
<tr>
<th>Table 3. Plasma insulin levels</th>
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<tr>
<td>Plasma Insulin, pg/ml</td>
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<tr>
<td>LETO</td>
</tr>
<tr>
<td>29 Wk</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>ID</td>
</tr>
<tr>
<td>PH</td>
</tr>
<tr>
<td>Values are means ± SE (n = 5–6). †P &lt; 0.05 and ††P &lt; 0.01 compared with each age of LETO control; **P &lt; 0.01 compared with OLETF control group at 43 wk of age.</td>
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Fig. 6. Pancreas insulin levels and histology. A: insulin levels of pancreas tissues by Western blotting. Values are means ± SE (n = 5–6). *P < 0.05 vs. LETO Cont; †P < 0.05 and ††P < 0.01 vs. each Cont group. B: representative image of histochemical analysis of insulin expression by anti-insulin antibody. Brown area indicates positive staining for insulin. C: fibrosis of the pancreas by Azan-Mallory stain. Blue areas correspond to fibrotic areas.
and PH in OLETF rats markedly decreased lipid peroxide levels. Plasma SOD-like activity was significantly lower in control OLETF rats than in control LETO rats (Fig. 4). The plasma SOD-like activity was higher in OLETF-ID rats than in control LETO rats. Superoxide generation in the liver of OLETF rats was much higher than that of LETO rats (Fig. 5). The ID diet markedly decreased superoxide generation in both LETO and OLETF rats, whereas PH in OLETF rats only moderately decreased superoxide production.

**Plasma and pancreatic insulin levels and pancreatic fibrosis.** Plasma insulin levels in OLETF rats were significantly higher than in LETO rats at 29 wk of age (Table 3). In contrast, insulin levels reversed the buildup at 43 wk of age. Similarly, insulin levels in the pancreas decreased markedly in the control OLETF rats (Fig. 6, A and B) with fibrosis of the pancreatic islets (Fig. 6C). The insulin level was maintained at normal levels in ID and PH OLETF rats (Table 3 and Fig. 6, A and B), whereas the LETO-ID and PH groups did not show any effect on insulin levels or histology compared with those of control LETO rats.

Gene expression of PPAR subtypes in pancreas and liver. mRNA expression of PPARα and PPARγ in the pancreas was decreased in ID rats compared with the appropriate control groups (Fig. 7). There were no significant differences in pancreatic PPARα mRNA levels between groups. However, PPARδ mRNA expression was suppressed in OLETF compared with control LETO rats, whereas PH significantly increased mRNA expression. In liver, mRNA expression of PPARα and PPARγ was significantly lower in OLETF rats than in LETO rats (Supplemental Fig. S2). ID did not affect the expression of either subtype. PPARδ mRNA did not vary between nondiabetic and diabetic animals, but its expression was increased by iron depletion.

Protein levels of PPARδ and HIF-1α in the pancreas. The protein expression of PPARδ (Fig. 8) and HIF-1α (Fig. 9) was decreased markedly in pancreatic tissue in control OLETF rats relative to LETO rats. Iron depletion significantly elevated the levels of these proteins in OLETF rats.
**TGFβ signal in the pancreas.** Pancreatic TGFβ1 gene expression and Smad2 protein phosphorylation were increased significantly in OLETF rats compared with LETO rats (Fig. 10). Iron depletion inhibited the increase of TGFβ1 and Smad2 phosphorylation.

**DISCUSSION**

In this study, we found that plasma ferritin levels in OLETF rats were markedly higher than in LETO rats. Iron depletion is beneficial in type 2 diabetic rats for managing diabetic parameters such as altered lipid and glucose metabolism. Iron depletion decreased fibrosis of pancreatic islet cells and increased mRNA and protein levels of PPARγ in the pancreas. Furthermore, these results suggest that iron depletion decreased oxidative stress throughout the whole body.

Iron deposition (Fig. 1) and the increase of plasma ferritin levels (Fig. 2) were observed in OLETF rats, and plasma, liver, and pancreatic lipid peroxidation was also increased (Fig. 3) in diabetic rats. It has been reported that changes in iron stores were monitored by plasma ferritin measurements (29). Thus, iron depletion may improve diabetic abnormalities by inhibiting ROS generation with the decreased labile iron pool. Furthermore, we found that iron depletion restored PPARγ expression in the pancreas. It is interesting to note that the nondiabetic LETO rats fed the ID diet had the lowest HDL and total cholesterol levels of all experimental groups (Table 2). Although the lower iron limits of these parameters were not defined, maintenance of iron stores in the body may be necessary for lipid homeostasis. In a recent study, gene array analysis of rat intestine revealed severe downregulation of apolipoprotein H in ID animals (34). Apolipoprotein H is present in TG-enriched lipoproteins (chylomicrons, VLDL, and LDL), and its role, still not completely characterized, was formerly related to the TG “cleanup” of plasma (35). Thus, our study and several other studies have demonstrated an association between iron deficiency and altered lipid metabolic status.

There are three subtypes of PPARs: PPARα, PPARβ/δ, and PPARγ. All three are sensors of dietary lipids and regulate different aspects of metabolic homeostasis and inflammatory responses (5, 17). However, in this study, the relationship during PPARγ expression in the pancreas and plasma ferritin and/or lipid levels could not be cleared. In the presence of insulin, the stimulation of NF-κB protein expression by glycated protein was decreased, whereas the expression of PPARγ protein was enhanced (4). Iron depletion maintained the plasma insulin levels in OLETF rats (Table 3). Therefore, preservation of insulin levels might be important for PPAR expression. PPARα is expressed mostly in the liver and heart, where it regulates fatty acid catabolism (12, 19). In contrast, PPARγ is expressed in adipose tissue and plays an important role in lipid storage and adipogenesis (1, 16, 28). PPARβ/δ is the predominant PPAR isoform in islets (2). Recently, it has been reported that the PPARγ agonist GW-501516 reverses multiple abnormalities associated with the metabolic syndrome by reducing oxidative stress and increasing fatty acid oxidation in moderately obese men (27). It has also been suggested that PPARδ may be a new therapeutic target to regulate heart reperfusion-associated oxidative stress and stimulation of enzymatic antioxidative defenses in rat cardiomyoblasts (25).

In this study, iron depletion decreased oxidative stress (Figs. 3 and 5) and increased pancreatic PPARδ mRNA expression.
(Fig. 7), resulting in the improvement of diabetic abnormalities. Although hepatic PPARα and PPARγ mRNA decreased in OLETF rats, iron depletion did not affect these PPAR subtypes. Taken together, these results suggest that iron depletion may primarily affect pancreatic function associated with decreased oxidative stress throughout the whole body. Furthermore, an iron chelator, deferoxamine, has been shown to stabilize HIF-1α expression, which increased glucose transporter expression (6). We also found that iron depletion could increase nuclear HIF-1α expression in the pancreas (Fig. 9). This effect may be one reason for the decrease in blood glucose.

In OLETF rats the plasma IL-6 protein level, which was inhibited by iron depletion, was increased (Table 2). A significant positive correlation between intestinal iron absorption and circulating IL-6 levels was also reported (14, 26). IL-6 per se, although it induces hepcidin mRNA, failed to affect basal iron absorption (26). A recent study also showed that the PPARδ agonist GW501516 dose-dependently suppressed IL-6-induced mRNA expression of acute-phase proteins (13). However, iron depletion increased pancreas PPARδ expression. Therefore, the diabetic symptoms might be improved by harnessing these synergistic actions. Iron depletion inhibited the pancreatic TGFβ signal, thus inhibiting the phosphorylation of Smad2. Therefore, the inhibition of the TGFβ pathway might inhibit pancreatic fibrosis. Figure 11 summarizes the pathways possibly affected by iron depletion in diabetic conditions.

In conclusion, iron depletion modulated insulin levels, oxidative stress, and pancreatic PPARδ expression and may offer a possible approach to overcome the negative outcomes associated with type 2 diabetes.

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DISCLOSURES

The authors declare that there is no duality of interest associated with this article.

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