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Iron deficiency attenuates catecholamine-stimulated lipolysis via downregulation of lipolysis-related proteins and glucose utilization in 3T3-L1 adipocytes

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Abstract. Iron deficiency has been associated with obesity and related metabolic disorders. The aim of the present study was to evaluate the effect of iron deficiency on fat metabolism, particularly regarding the lipolytic activity, lipolysis-related protein expression, and glucose utilization of adipocytes. Differentiated 3T3-L1 adipocytes were incubated with an iron chelator, deferoxamine mesylate (DFO), for 48 h. Subsequently, basal and isoproterenol-stimulated lipolytic activities, the proteins involved in lipolysis and glucose utilization were compared with a control (CON). The results revealed that treatment with DFO significantly decreased the free iron content but did not affect total protein and lipid contents in adipocytes. Iron deprivation caused a significant reduction in isoproterenol-stimulated lipolysis, but not basal lipolysis. Lipolysis-related proteins, including perilipin A, adipose triglyceride lipase, hormone sensitive lipase and comparative gene identification-58, were decreased in the DFO compared with the CON group. Furthermore, glucose utilization, a major precursor of 3-glycerol phosphate for micro-lipid droplet synthesis during lipolysis and the expression of glucose transporter (GLUT) 4 were significantly lower in the DFO group when compared with the CON group. However, hypoxia-inducible factor-1α and GLUT1 expressions were upregulated in DFO-treated adipocytes. In conclusion, the results indicated that low iron availability attenuated catecholamine-stimulated lipolysis by downregulating lipolytic enzymes and glucose utilization in 3T3-L1 adipocytes.

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Abbreviations: DFO, deferoxamine mesylate; GLUT, glucose transporter; mLD, micro-lipid droplet

Key words: obesity, lipid metabolism, glucose transporter, hypoxia, mitochondria

Introduction

Obesity is defined as abnormal or excessive fat accumulation in adipose tissue, and results from an imbalance between energy intake and expenditure. The excess dietary free fatty acids are esterified to inert triglycerides, which are stored in lipid droplets in adipose tissue. When energy demand is increased, triglycerides are hydrolyzed to free fatty acids and glycerol. This process is called lipolysis. Lipolysis is regulated by specific hydrolases and its activators. Adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) are the major lipases. ATGL deficiency in mice is associated with severely reduced lipolysis, resulting in increased fat deposition (1), suggesting the important role of ATGL in triglyceride catabolism. Furthermore, deficiency or dysfunction of a potent ATGL coactivator, comparative gene identification-58 (CGI-58), also causes severe systemic triglyceride accumulation in mice and human patients (2,3). Perilipin A coats lipid droplets and regulates triglyceride hydrolysis by inhibiting the access of lipases into lipid droplets. Obesity is associated with reduced lipolytic activity and lipolysis-related proteins in adipose tissue (4,5). Thus, understanding the regulatory mechanisms of lipolytic activity and lipolysis-related proteins is important to develop strategies against metabolic disorders.

Dysregulation of iron status has been shown to be linked to the development of obesity-related metabolic disorders, such as type 2 diabetes. The potential links between iron and metabolic disorders are hypothesized to an induction of inflammation and oxidative stress due to the iron overload (6,7). In contrast, previous studies have reported that the etiology of obesity-associated iron deficiency includes inadequate dietary iron intake, increased iron requirements, and menstrual irregularities (8-12); however, the precise mechanisms are unclear. Hemoglobin concentration or liver iron storage have been reported to be inversely related to body fat in a rodent model and humans (13,14). As lipolytic activity is dysregulated in adipose tissue by obesity as mentioned above, it is possible that not only accumulation of iron, but iron deficiency affects lipolytic activity in adipose tissue. Yamagishi *et al* (15) reported that rats fed iron deficient diet for 1 week shows the increased catecholamine-stimulated lipolytic activity in epididymal

adipocytes. However, the elevation of lipolytic activity by iron deficient diet was not observed rats fed the diet for 5 weeks. Furthermore, the influence of iron availability in the regulation of lipolysis-related proteins, such as ATGL, HSL or perilipin A in adipocytes were not evaluated in that study.

Recent evidence obtained from 3T3-L1 adipocytes revealed that micro-lipid droplets (mLDs) play an important role in active lipolysis (16,17). During mLD formation, glycerol is derived from glucose through glycolysis to synthesize triglycerides, because of the lack of glycerol kinase in adipocytes. It is well known that glucose is preferred over lipid as a substrate under iron deficiency (18,19). Therefore, changes in glucose utilization induced by iron deficiency may influence lipolytic activity in adipocytes.

To evaluate these possibilities, we treated 3T3-L1 adipocytes with a hydrophilic chelator, deferoxamine (DFO) used in clinical practice to remove excess iron. Here, we report that DFO-induced iron deficiency reduces lipolytic activity through downregulation of lipolysis-related proteins and glucose utilization in adipocytes.

Materials and methods

Cell culture. All reagents for cell culture were obtained from Nacalai Tesque, unless otherwise indicated. 3T3-L1 cells were purchased from JCRB Cell Bank. Cells were cultured as previously reported (20).

Experimental design. 3T3-L1 cells were maintained in growth medium (GM) containing DMEM, 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. For differentiation, confluent cells were treated with a hormone mixture containing 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 μg/ml insulin in GM. After 48 h, the hormone mixture was removed and cells were further cultured in GM supplemented with 1 μg/ml insulin. At 7 days after differentiation induction, 3T3-L1 adipocytes were treated with deferoxamine mesylate (DFO) (Sigma-Aldrich; Merck KGaA) at the indicated concentrations for 48 h. At day 9, the cells in all groups were analyzed.

Lipolytic stimulation. Lipolytic stimulation was applied to differentiated 3T3-L1 adipocytes as follows. After washing twice with PBS, the cells were incubated with phenol red-free DMEM containing 2% BSA in presence or absence of 1 μM isoproterenol at 37˚C for up to 4 h. To inhibit glucose uptake, the cells were treated as above, except that 500 μM cytochalasin B (Sigma-Aldrich; Merck KGaA) was added to the medium 30 min before lipolysis stimulation. Aliquots of the medium were collected as specified time points, and glycerol and glucose concentrations were measured using a glycerol assay kit (Sigma-Aldrich; Merck KGaA) and a Glucose CII test wako (Wako), respectively.

Oil Red O staining. 3T3-L1 adipocytes were grown in 24-well plates. Differentiated cells at day 9 were washed twice with PBS and fixed with 4% PFA for 30 min. Oil Red O stain stock solution was diluted at a 6:4 ratio with distilled water and then filtered. Cells were stained with the Oil Red O solution for 30 min at room temperature. The stained cells were washed with distilled water three times and observed under a microscope (Keyence). To quantify the lipid content, the Oil Red O was eluted with 100% isopropanol for 10 min, and the absorbance at 490 nm was measured using a microplate reader (Bio-Rad Laboratories, Inc.).

Cellular iron concentration measurement. 3T3-L1 adipocytes were grown in 6-well plates as described above. Differentiated cells at day 9 were washed with PBS twice and lysed in protein precipitation solution containing 0.53 N HCl, 5.3% trichloroacetic acid. The crude lysate was boiled at 100˚C for 30 min, then the sample was allowed to slowly cool down to room temperature. The lysate was centrifuged at 15,000 x g for 5 min, and the supernatant was used to measure iron concentration. The Iron Assay kit (Metallogenics) was used to measure cellular iron content according to the manufacturer's protocol

Western blotting. 3T3-L1 adipocytes were grown in 12-well plates as described above, washed with PBS twice, and then harvested in ice-cold radioimmunoprecipitation assay buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA, and protease/phosphatase inhibitor cocktail. Cell lysates were kept on ice for 10 min, then centrifuged at 15,000 x g for 10 min. The supernatants were collected and protein concentrations were measured using a bicinchoninic acid assay kit. Samples were prepared in 4X Laemmli sample buffer. Equal amounts of sample protein were separated by 7.5 or 12.5% SDS-PAGE, transferred to a PVDF membrane (#88518; Pierce; Thermo Fisher Scientific, Inc.), and incubated overnight at 4˚C with primary antibodies. Primary antibodies are listed in Table SI. Enhanced chemiluminescence (Merck KGaA) was used to facilitate the detection of protein bands. Images were scanned using a chemiluminescence detector (LAS500; GE Healthcare Bio-Sciences AB). Band intensities were quantified using ImageJ 1.52a (National institutes of Health). Equal loading was checked by staining the blot with Coomassie Brilliant Blue (#296-21541; Wako) (16,17).

Statistical analysis. Data are expressed as means ± standard errors of the mean. Statistical analyses were carried out using BellCurve for Excel version 3.10 (Social Survey Research Information, Tokyo, Japan). Differences between groups were assessed by one- or two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test, or unpaired t-tests, as indicated. $P<0.05$ was considered to indicate a statistically significant difference.

Results

DFO treatment does not affect lipid and protein contents in 3T3-L1 adipocytes. We first examined whether iron deficiency caused by the iron chelator DFO affects lipid storage in 3T3-L1 adipocytes. As shown in Fig. 1A and B, lipid content in 3T3-L1 adipocytes did not differ between the treatment groups ($P=0.674$). The protein content of PPARγ, a marker of adipocyte differentiation, was not different among the groups ($P=0.176$; Fig. 1C and D). In addition, there was no difference in total protein content between the groups ($P=0.531$; Fig. 1E). DFO treatment at 500 μM significantly decreased cellular iron

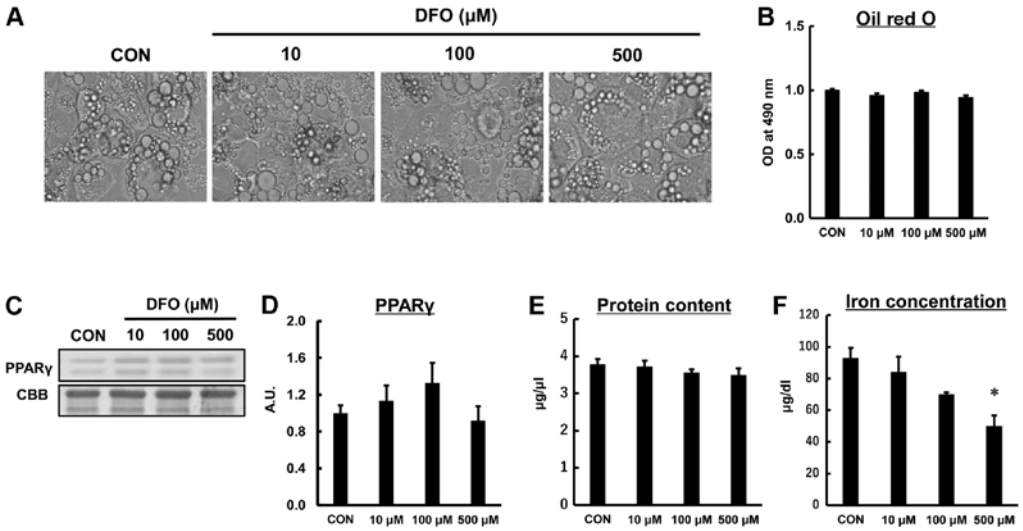

Figure 1. Effects of iron deficiency on triglyceride accumulation, PPARγ/total protein content, and intracellular iron concentrations in differentiated 3T3-L1 cells. (A) Optical microscopy images of adipocytes in the different DFO treatment groups (magnification, x40). (B) Differentiated 3T3-L1 adipocytes were fixed and stained with Oil Red O to evaluate lipid content. Stained lipids were quantified by measuring absorbance at 490 nm. (C) Representative western blots for PPARγ. CBB was used as a loading control. (D) Level of PPARγ protein. (E) Total protein content in adipocytes was measured using a bicinchoninic acid assay. (F) Intracellular iron concentration. One-way ANOVA was used for statistical analysis (n=3-6). *P<0.05 vs. CON. PPARγ, peroxisome proliferator-activated receptor γ; DFO, deferoxamine mesylate; CBB, coomassie brilliant blue; OD, optical density; CON, control; A.U., arbitrary units.

concentration compared to the control group (P<0.05; Fig. 1F). These results suggested that the protocols used in the present study reduces the iron concentration in adipocytes, but did not affect lipid content or cause maladaptive responses.

Iron deficiency reduces catecholamine-stimulated, but not basal lipolytic activity in 3T3-L1 adipocytes. Next, we investigated the basal and catecholamine-stimulated lipolytic activities in DFO-treated adipocytes. Glycerol release into the medium during lipolysis in the presence or absence of catecholamine was measured for 4 h. The amount of glycerol, which represents total lipolytic activity in adipocytes, was increased in cells treated with 500 μM DFO at basal state compared to the CON group (P<0.01; Fig. 2A). In contrast, under catecholamine-stimulated lipolysis, DFO treatment at 100 μM significantly decreased glycerol release when compared to the control group (P<0.01), and a further decrease in glycerol release was observed in the 500 μM group (P<0.01; Fig. 2B).

Iron deficiency decreases lipolysis-related proteins in 3T3-L1 adipocytes. Lipolysis-related proteins, including ATGL, HSL, perilipin A and CGI-58, play important roles in regulating fat storage and breakdown (16,21-23). Given the decrease in catecholamine-stimulated lipolysis activity due to 2-day iron deficiency, we hypothesized that iron deficiency would suppress lipolysis-related proteins. Therefore, we investigated the effect of iron deficiency on lipolysis-related protein contents. As shown in Fig. 3, DFO treatment decreased the levels of ATGL, HSL, CGI-58, and perilipin A proteins in a concentration-dependent manner. Hypoxia inducible factor (HIF)-1α was induced by DFO treatment (Fig. 3F).

Iron deficiency modulates glucose transporters and glucose utilization during lipolysis in 3T3-L1 adipocytes. GLUT1, a transporter that plays an important role in basal glucose uptake, was strongly induced by iron deficiency (Fig. 4B). However, the protein content of the insulin-responsive glucose transporter GLUT4 was decreased by DFO treatment in a dose-dependent manner (Fig. 4C). The decrease rate of the glucose concentration in the medium, which represents glucose utilization of adipocytes, was lower in DFO-treated than control cells (Fig. 4D).

To evaluate the effect of glucose availability on lipolytic capacity in adipocytes, the cells were pretreated with cytochalasin B before lipolysis stimulation. Pretreatment with cytochalasin B resulted in higher glucose concentrations in the culture medium 4 h after lipolysis stimulation, indicating low glucose utilization (Fig. 4E). The glycerol concentration in the medium was lower in cytochalasin B-treated adipocytes (Fig. 4F), implying that inhibition of glucose utilization reduces catecholamine-stimulated lipolysis.

Short-term iron deficiency does not affect mitochondrial proteins. The protein content of NDUFB8, a subunit of mitochondrial complex I containing iron, did not differ between the treatment groups (CON, 1.00±0.15 arbitrary unit; 10 μM DFO, 0.92±0.13; 100 μM DFO, 0.91±0.23; 500 μM DFO, 0.91±0.21). The non-iron-containing mitochondrial proteins citrate synthase (CON, 1.00±0.10 arbitrary unit; 10 μM DFO, 1.06±0.11; 100 μM DFO, 1.13±0.1; 500 μM DFO, 0.95±0.01) and long-chain acyl-CoA dehydrogenase (CON, 1.00±0.03 arbitrary unit; 10 μM DFO, 0.90±0.10; 100 μM DFO, 0.88±008; 500 μM DFO, 0.84±0.11) were also unaffected by DFO treatment.

Discussion

Although iron deficiency is frequently found in advanced stages of obesity, the precise mechanisms by which obesity status causes iron deficiency are unclear (24). It was reported that catecholamine-stimulated lipolysis increased in epididymal white adipocytes isolated from rats fed iron deficient

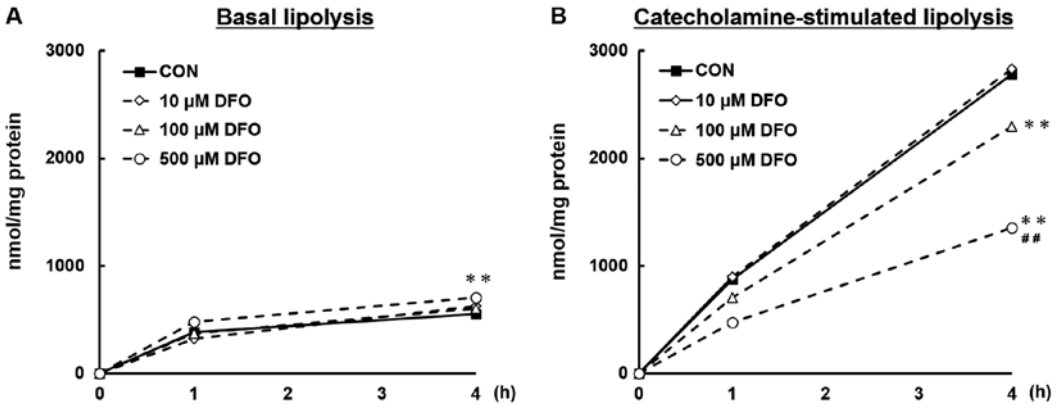

Figure 2. Effect of iron deficiency on basal and catecholamine-stimulated lipolysis. Glycerol release into the medium was measured under (A) basal and (B) catecholamine-stimulated conditions. Two-way ANOVA was used for statistical analysis (n=5-6). **P<0.01 vs. CON; $^{\#\#}$P<0.01 vs. 10 μM DFO. CON, control; DFO, deferoxamine mesylate.

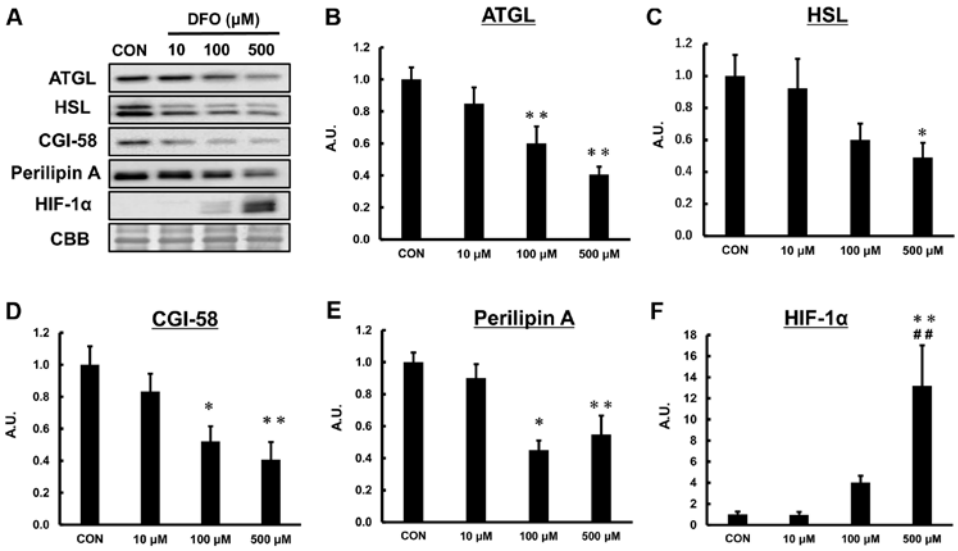

Figure 3. Effect of iron deficiency on adipocyte lipid-related proteins. (A) Representative western blot analysis for (B) ATGL, (C) HSL, (D) CGI-58, (E) perilipin A and (F) HIF-1α. CBB was used as a loading control. One-way ANOVA was used for statistical analysis (n=5-6). *P<0.05 and **P<0.01 vs. CON. $^{\#\#}$P<0.01 vs. 10 μM DFO. ATGL, adipose triglyceride lipase; HSL, hormone sensitive lipase; CGI-58, comparative gene identification-58; HIF-1α, hypoxia inducible factor-1α; CON, control; DFO, deferoxamine mesylate; CBB, coomassie brilliant blue; A.U., arbitrary units.

diet for 1 week. However, in that study, the effect of iron deficient diet on lipolytic activity wore off when the diet was continued for 5 weeks (15), and effect of iron deficiency on lipolysis-related proteins were not evaluated. In this study, we used 3T3-L1 adipocytes to investigate the molecular regulation of lipolysis in iron deficient state in 3T3-L1 adipocytes and found that DFO-induced iron deficiency results in decreases in lipolysis-related proteins, including perilipin A, ATGL, HSL, and CGI-58. Consequently, catecholamine-stimulated lipolytic activity was reduced in 3T3-L1 adipocytes treated with DFO in a dose-dependent manner. These results suggested that low iron availability attenuates lipolytic activity through the reduction of lipolysis-related proteins in adipocytes.

During active lipolysis, numerous mLDs, which play an important role in lipolysis, appear in all areas of the cytoplasm (16). For the formation of mLDs, glucose utilization becomes active to provide glycerol-3-phosphate, a partner of fatty acid, to form triglyceride during lipolysis. Our results demonstrated that iron deficiency causes a decrease in GLUT4 protein content, concomitant to reduced glucose utilization. These results indicated that not only lipolytic enzymes, but also glucose utilization capacity contributes to the reduction in catecholamine-stimulated lipolytic activity in DFO-treated cells.

Previous reports have shown that GLUT1 and GLUT4 respond differently to physiological stimuli in 3T3-L1 adipocytes and human adipose tissue (25-27). We observed an induction of GLUT1 and HIF-1α protein contents by DFO treatment, which is consistent with a previous study showing that hypoxic stimulation induces GLUT1 in 3T3-L1 adipocytes (28). In contrast, the protein content of the insulin-responsive glucose transporter, GLUT4, was decreased in DFO-treated cells. These results indicated that GLUT4 and glucose play a critical role in catecholamine-stimulated lipolytic activity, and induction of GLUT1 by iron deficiency is not sufficient to compensate the reduction in GLUT4. Because there is a hypoxia-responsive element in the *GLUT1*

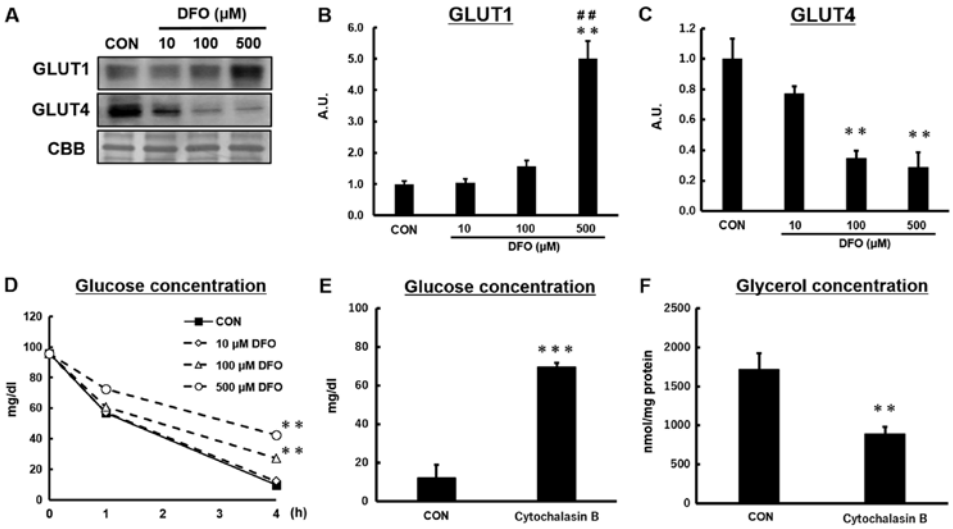

Figure 4. Effect of iron deficiency on glucose transporters and glucose utilization. (A) Representative western blot analysis of GLUT1 and GLUT4. CBB was used as a loading control. Protein contents of (B) GLUT1 and (C) GLUT4 as measured by western blotting. One-way ANOVA was used as statistical test. (D) A decline in glucose concentration was demonstrated in medium. Concentrations are representative of glucose utilization in adipocytes during isoproterenol-stimulated lipolysis. Two-way ANOVA was used as statistical test. (E) Glucose and (F) glycerol concentrations in medium after 4 h isoproterenol-stimulated lipolysis in the presence or absence of the glucose transporter inhibitor, cytochalasin B. An unpaired t-test was used for statistical analysis (n=5-6). **P<0.01 and ***P<0.001 vs. CON; $^{\#\#}$P<0.01 vs. 10 μM DFO. GLUT, glucose transporter; CBB, coomassie brilliant blue; DFO, deferoxamine mesylate; CON, control; A.U., arbitrary units.

promoter, the induction of GLUT1 by DFO is reasonable. It is difficult to explain the mechanisms underlying the reduction in GLUT4 expression in this study, although some other studies have also reported such a decrease in GLUT4 in response to hypoxia (26-28). In this study, we measured the total protein content of GLUT1 and GLUT4 in adipocytes. It is well known that GLUTs translocate from the intracellular pool to plasma membranes and elevate glucose transport activity by physiological stimuli. Therefore, the distribution of GLUTs in response to catecholamine stimulation and iron deficiency should be examined in future studies.

When adipocytes become hypertrophic during the development of obesity, oxygen cannot diffuse into cells, which results in hypoxia. As shown in Fig. 3F, DFO treatment induced HIF-1α, indicating that low iron availability causes a hypoxic state in adipocytes. Iron deficiency has been shown to induce hypoxia in various cell types. As carbohydrate is preferred as an energy source over fat under hypoxia, the hypoxic state is one possible mechanism explaining the reduction in lipolysis-related proteins in DFO-treated adipocytes. However, the molecular mechanisms by which an iron deficiency-induced hypoxic state downregulates lipolysis-related proteins remains unknown. In addition, there is no direct evidence that the hypoxic state observed in adipose tissue from obese subjects is due to iron deficiency. Further *in vivo* study is needed to determine whether iron deficiency is responsible for the hypoxic state and the reduction in lipolytic capacity in obese adipose tissue. In addition, the responses to physiological stimuli that activate lipolysis, such as catecholamine or starvation, should be examined *in vivo*.

There are conflicting reports showing that hypoxia increases or decreases lipid accumulation in adipocytes (26,29-33). Although HIF-1α was elevated in DFO-treated adipocytes in this study, there was no difference in lipid concentration between the groups. This might be due to the short-term treatment with DFO. For example, Marques *et al* (26) reported an increment in lipid accumulation in adipocytes treated with $CoCl_2$, a hypoxia mimetic, or DFO for 7 days. In contrast, we treated adipocytes with DFO for 2 days, which is a shorter treatment time than that in previous studies, because it has been reported that hypoxia attenuates adipocyte differentiation via suppression of PPARγ (34). To exclude the effect of iron deficiency on adipocyte differentiation, we started DFO treatment at day 7 after differentiation initiation, when the formation of large lipid droplets was visible. DFO treatment had no effect on PPARγ protein content, suggesting that DFO-induced reduction in lipolysis-related proteins was not due to inhibition of adipocyte differentiation.

To support our results, we further incubated differentiated 3T3-L1 adipocytes with iron sulfate, and found that iron overload causes a significant reduction of lipid droplet size (data not shown). This could be because of oxidative stress caused by excess free iron. It was reported that iron overload induces a decrease in fat mass in C57BL/6 mice fed a high-iron diet for 8 weeks (35). These results led us to consider that iron overload model is suitable for evaluation of adipocyte differentiation, but not for lipolysis. Establishment of a suitable model for iron overload is needed to evaluate the effect of iron accumulation on lipolysis in adipocytes.

Mitochondrial dysfunction attenuates lipolysis in adipocytes (36). Inhibition of the electron transport chain abolishes lipolysis stimulated by catecholamine (37). Iron deficiency has been shown to reduce mitochondrial proteins that contain iron as a cofactor in several cell types (19,38,39). Based on these findings, we asked whether mitochondrial dysfunction is a primary cause of reduced catecholamine-stimulated lipolysis in adipocytes treated with DFO. However, contents of both iron-containing (complex I subunit) and non-iron-containing protein (citrate synthase and LCAD) did not differ between

treatment groups. These results suggest that the iron deficiency-induced decrease in catecholamine-stimulated lipolysis occurs independently of or prior to mitochondrial dysfunction in adipocytes. The molecular mechanism by which iron deficiency attenuates lipolysis without a reduction in mitochondria proteins remains unknown.

In conclusion, our results suggest that low iron availability attenuates catecholamine-stimulated lipolysis in 3T3-L1 adipocytes. This reduction is due to decreases in lipolysis-related proteins and glucose utilization. Taking these results together, the present study extends our understanding of the importance of iron in adipocyte biology and lipid metabolism. Further studies are required to reveal how iron deficiency causes hypoxia in adipose tissue and stimulates the metabolic programing observed in this study.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KH and NN designed and performed the experiments, analyzed the data and wrote the manuscript. KH, NT and SI performed the experiments and analyzed the data. TH designed the study, analyzed the data and wrote the manuscript. KH, NT, SI, TH and NN discussed the results, commented on the manuscript and approved the manuscript for submission.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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