Research article

Iron deficiency negatively regulates protein methylation via the downregulation of protein arginine methyltransferase

Hirofumi Inoue [a], Nobuaki Hanawa [b], Shin-Ichi Katsumata [b], Yumi Aizawa [c], Rie Katsumata-Tsuboi [a], Miori Tanaka [a], Nobuyuki Takahashi [a], Mariko Uehara [a,*]

[a] Department of Nutritional Science and Food Safety, Faculty of Applied Bioscience, Tokyo University of Agriculture, Japan
[b] Department of Nutritional Science, Faculty of Applied Bioscience, Tokyo University of Agriculture, Japan
[c] Department of Agricultural Chemistry, Faculty of Applied Biosciences, Tokyo University of Agriculture, Tokyo, Japan

A B S T R A C T

Iron is an essential trace metal for all biological processes and plays a role in almost every aspect of body growth. Previously, we found that iron-depletion downregulated the expression of proteins, arginine methyltransferase-1 and 3 (PRMT1 and PRMT3), by an iron-specific chelator, deferoxamine (DFO), in rat liver FAO cell line using DNA microarray analysis (unpublished data). However, regulatory mechanisms underlying the association between iron deficiency and PRMT expression are unclear *in vitro* and *in vivo*. In the present study, we revealed that the treatment of cells with two iron-specific chelators, DFO and deferasirox (DFX), downregulated the gene and protein expression of PRMT1 and 3 as compared with the untreated cells. Subsequently, DFO and DFX treatments decreased protein methylation. Importantly, these effects were attenuated by a holo-transferrin treatment. Furthermore, weanling Wistar-strain rats were fed a control diet or an iron-deficient diet for 4 weeks. Dietary iron deficiency was found to decrease the concentration of hemoglobin and liver iron while increasing the heart weight. PRMT and protein methylation levels were also significantly reduced in the iron-deficient group as compared to the control group. To our knowledge, this is the first study to demonstrate that PRMT levels and protein methylation are reduced in iron-deficient models, *in vitro* and *in vivo*.

1. Introduction

Iron is one of the essential trace metals for all biological processes such as regulation of regulating DNA replication, cell proliferation and cell growth, and plays an important role in the redox system in almost tissues [1, 2, 3]. The levels of intracellular iron are strictly regulated by iron transporters and iron-binding proteins under normal physiological conditions [4]. In contrast, excess iron causes oxidative stress because of an increase in the hydroxyl radicals via the Fenton/Haber–Weiss catalytic reaction, which causes tissue damage [5].

Iron deficiency is a nutritional problem face by more than two billion people, mainly women and children, in the world [6]. Several studies have suggested that iron deficiency is as a common cause of anemia and has been recognized as a complication of chronic conditions such as inflammatory bowel disease, rheumatoid arthritis, and chronic kidney disease [7, 8, 9]. In particular, the iron status of an individual may play

an important role in body health not only via excess iron but also via iron deficiency. Both iron-excess and -deficiency can lead to significant problems. Our research group previously reported that phosphatidylcholine hydroperoxide, a primary peroxide of biological membranes, is increased in iron-deficient rat livers with copper accumulation [10]. Furthermore, iron deficiency induces osteoporosis and induction of reactive oxygen species (ROS) *in vitro* and in *in vivo* models [11, 12]. Therefore, the health consequences of iron deficiency-induced anemia are manifold and include increased mortality rates and decreased physical capacities.

Our previous study using microarray analysis (GEO, Series ID GSE109656) revealed that the treatment of rat liver cell line, FAO cells, with iron-specific chelator deferoxamine (DFO) downregulated 1,859 genes (fold-change > 1.5) compared to untreated cells [13]. Thus, antioxidant molecules including the NAD(P)H dehydrogenase quinone 1 and glutathione peroxidase protein families were downregulated by DFO

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treatment. Interestingly, we found that methionine metabolism-associated genes and proteins of arginine methy transferases (PMRTs), which are involved in post-translational protein methylation, were also downregulated in DFO-treated cells (unpublished data). Thus, we focused on the effects of iron-depletion on the protein methylation by PRMTs in FAO cells.

PRMTs are known to regulate many cellular processes such as epigenetic gene modifications and expression, RNA processing, DNA repair, signal transduction, and post-translational protein methylation [14, 15]. In addition, PRMTs can catalyze the formation of monomethyl arginine and either asymmetric or symmetric dimethyl arginines [16]. Type I PRMTs catalyze the formation of monomethyl-arginine and asymmetric dimethyl arginine (ADMA), whereas type II enzymes form monomethylarginine and symmetric dimethyl arginine. The type I methyltransferases include PRMT1, PRMT3, PRMT4, PRMT6, and PRMT8, while the type II enzymes include PRMT5, PRMT7, and PRMT9 [17]. However, the effects of iron-depletion on PRMT expression and protein methylation remain unclear. In the present study, we examined the effects of iron-depletion on the protein methylation by down-regulating PRMT1 and 3 *in vitro* and *in vivo*.

2. Materials and methods

2.1. Reagents

FAO cells were treated with the iron-specific chelator deferoxamine (DFO: Santa Cruz Biotechnology, Dallas, TX, USA) or deferasirox (DFX: Cayman Chemical, Ann Arbor, Michigan, USA) was purchased, respectively. Antibodies against anti-PRMT1, anti-PRMT3, ADMA and anti-β-actin were purchased from Santa Cruz Biotechnology.

2.2. Cell culture

FAO cells were cultured in DMEM/F-12 Ham's (Gibco BRL/Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Biowest, Nuaillé, France), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL/Invitrogen) at 37 °C in 5% CO_2.

2.3. Cell viability assay

FAO cells (1×10^5 cells/well) were cultured with 96-well plates and then cells were treated with various concentrations of DFO or DFX for 24 h in DMEM/F-12 Ham's containing 10% FBS. After treated cells were washed twice with phosphate-buffered saline (PBS) at room temperature, the cell viability was assessed using cell counting kit-8 (CCK-8: DOJINDO, Kumamoto, Japan). The effect of DFO or DFX on cell viability was expressed as percent cell viability, with vehicle-treated control cells set at 100%.

2.4. Animals

Twelve 3-week-old male Wistar rats were purchased from Clea Japan (Tokyo, Japan) and housed individually in metabolic cages at 22 °C with a 12-h light/dark cycle. The Tokyo University of Agriculture Animal Use Committee approved the study protocol, and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals of the university. All rats were fed a control diet for 3 d of acclimatization period. After 3 d, all rats were randomly divided into two experimental groups of six each and fed the control (CTL) diet or an iron-deficient (ID) diet. The experimental diets were based on the AIN-93G diet [11]. All rats were allowed access to food ad libitum and were given free access to distilled water for 4 weeks. After 4 weeks of the experimental period, all rats were sacrificed, and blood and liver samples were collected for analysis. Part of the blood sample was used to measure hemoglobin concentration using the hemoglobin B-test (Wako Pure

Chemical Industries, Osaka, Japan). Hearts were removed and weighed because heart enlargement is a sign of iron-deficient anemia.

2.5. Liver iron concentration

Liver samples were dried, weighed, and milled. Milled samples were demineralized with a 1 mol/L HCl solution. Iron concentration in liver were analyzed by atomic absorption spectrophotometry (Hitachi A-2000, Tokyo, Japan) according to the method of Gimblet *et al.* [18].

2.6. RNA extraction and real-time PCR

Total RNA was isolated from FAO cells or rat liver tissues using the Sepasol-RNA I Super G (Nacalai Tesque, INC., Kyoto, Japan). Next, cDNA was synthesized from total RNA using a PrimeScript RT reagent Kit (Takara Biotechnology, Shiga, Japan). Furthermore, cDNA from *in vivo* and *in vitro* were analyzed by real-time PCR to determine mRNA expression. Real-time PCR was performed on an ABI Step one Plus System (Applied Biosystems, Foster City, CA, USA) using THUNDERBIRD qPCR Mix (Toyobo, Osaka, Japan). The following primer sets were used: *PRMT1* (forward:5′-GGGAAGCTTTGGGTGGCCTGTT-3′, reverse:5′-TCGGCGCTCATCCCT-3′), *PRMT3* (forward:5′-GGGAAGCTTTGGGTGGCCTGTT-3′, reverse:5′-TCGGCGCTCATCCCT-3′), *GAPDH* (forward:5′-CGGCACAGTCAAGGCTGA-GAATG-3′, reverse:5′-TGAAGACGCCAGTAGACTCCACGAC-3′). All reactions were normalized to the housekeeping gene *GAPDH*.

2.7. Western blotting

FAO cells were washed twice with PBS and lysed with RIPA buffer (10 mM Tris-HCl, pH7.6, 150 mM sodium chloride, 0.1%(*w/v*) sodium deoxycholate, 0.1%(*w/v*) sodium dodecyl sulfate, 1.0%(*w/v*) NP-40 substitute). Livers were homogenized in homogenization buffer (10 mM HEPES (pH7.6, 1 mM EDTA, 250 mM sucrose, and phosphatase and protease inhibitor cocktails). Those samples were separated by SDS-PAGE, transferred to PVDF membrane. The membranes were blocked with 3% bovine serum albumin in Tris-buffered saline with 0.05% Tween-20 (TBST), and then immunoblot with anti-PRMT1 (1:1000), anti-PRMT3 (1:1000), anti-ADMA (1:1000) and β-Actin (1:1000) followed by HRP-conjugated anti-rabbit/mouse IgG antibodies and visualized using the EzWestLumi plus (ATTO, Tokyo, Japan).

2.8. Statistical analysis

Results are presented as the mean ± S.E. All data are the means of three independent experiments and multiple comparisons were performed with Tukey's test after one-way analysis of variance (ANOVA) for *in vitro* study. Student's t-test was performed for *in vivo* study. Unmatched letters denote significant differences, p < 0.05.

3. Results

3.1. Iron deficiency downregulated PRMT1 and PRMT3 gene expressions in FAO cells

FAO cells were treated with various concentrations of DFO or DFX, and the cell viability was evaluated using CCK-8 assay. DFO did not change the cell viability compared to untreated cells (Figure 1A). In contrast, high-doses (100 μM) of DFX induced cytotoxicity but not low-doses (Figure 1B). This assay indicated that the maximum concentration used in our subsequent studies (DFO:50 μM or DFX:100 μM) showed no cytotoxic effects in FAO cells. Furthermore, to demonstrate that DFO or DFX induced the downregulation of *PRMT1* and *PRMT3* expressions, we quantified the mRNA levels in DFO- or DFX-treated FAO cells by real-time PCR. Compared with untreated cells, both iron-specific chelators significantly downregulated the expression of *PRMT1* and *PRMT3* in a dose-dependent manner (Figure 1C and D). Taken together, these results
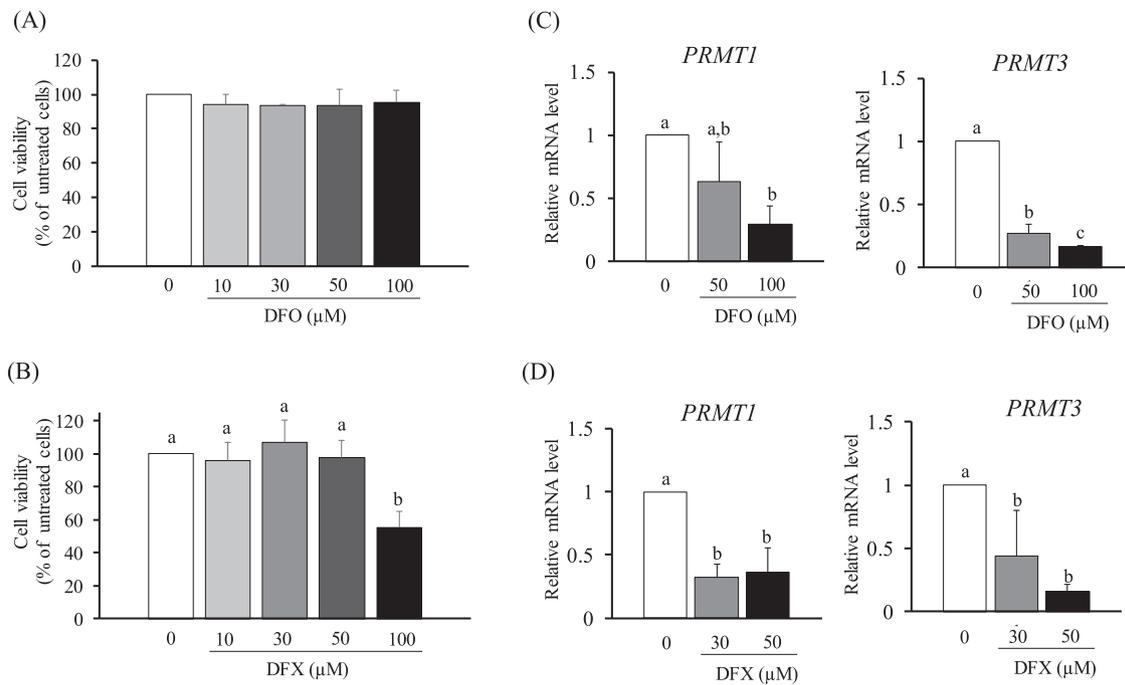
Figure 1. Effects of DFO or DFX treatment on gene expression of *PRMTs*. Cell viability of (A) DFO (0–100 μM) and (B) DFX (0–100 μM) in FAO cells for 24 h. (C and D) Total RNA of FAO cells was extracted and cDNA was synthesized. Then, the gene expression levels of *PRMT1* and *PRMT3* were quantified by real-time PCR with *GAPDH* serving as the internal standard. Data are shown as the means ± S.E. from at least 3 independent experiments. Multiple comparisons were performed using Tukey's test after one-way ANOVA. Unmatched letters denote significant differences, $p < 0.05$.

suggest that downregulation of *PRMT1* and *PRMT3* under iron-deficient conditions may decrease protein methylation.

3.2. Iron-deficient condition decreased PRMT1 and PRMT3 protein expressions and ADMA formation in FAO cells

Generally, PRMT1 and PRMT3 catalyze ADMA formation, which is protein methylation [17]. Therefore, we examined DFO- and DFX-treated cells to elucidate whether the iron-specific chelators decreased ADMA formation by downregulating *PRMT1* or *PRMT3*. After incubation with

DFO or DFX for 24 h, cell lysates were immunoblotted using anti-ADMA antibody. Treatment with DFO or DFX for 24 h decreased protein expressions of PRMT1 and PRMT3 and ADMA formation (Figure 2A and B). The downregulation of protein expressions was in parallel with the gene expressions of PRMTs. These phenomena indicated that iron-deficient conditions negatively regulate protein methylation by downregulating PRMT1 and PRMT3.
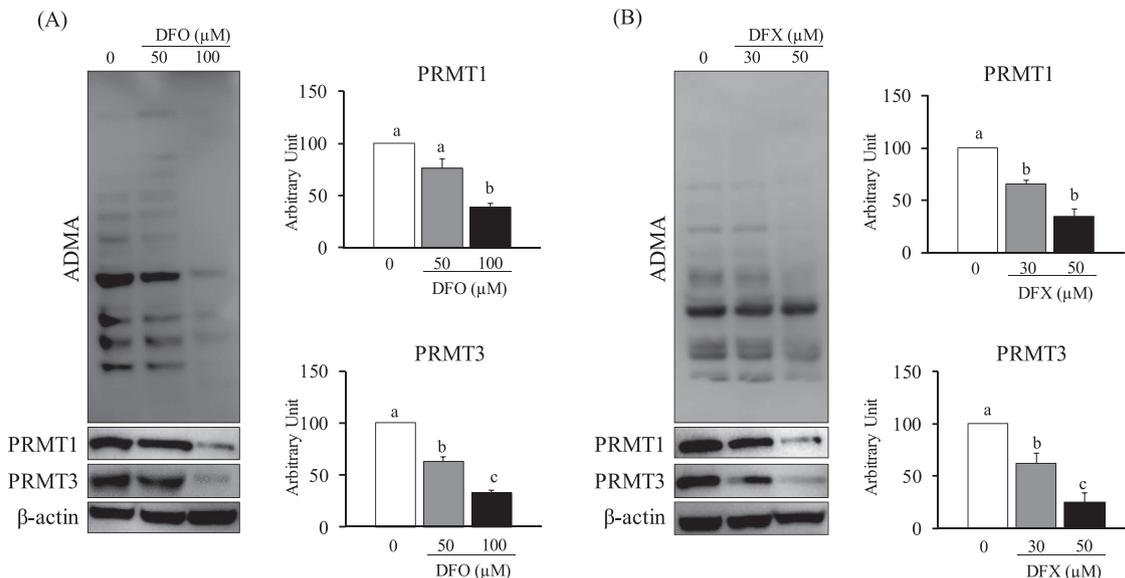
Figure 2. DFO or DFX treatment downregulated PRMTs protein and protein methylation. The expression FAO cells was exposed to (A) DFO or (B) DFX for 24 h. The cell lysates were immunoblotted with the indicated antibodies. β-Actin was used as an internal control. Quantification of PRMT1 or PRMT3 was performed by densitometry using Image J. Protein levels were normalized to β-Actin. Data are shown as the means ± S.E. from at least 3 independent experiments. Multiple comparisons were performed using Tukey's test after one-way ANOVA. Unmatched letters denote significant differences, $p < 0.05$. The full uncropped images are available as supplementary material.

3.3. Holo-transferrin restored decreasing PRMT1 and PRMT3 protein expressions and ADMA formation due to iron-deficient conditions in FAO cells

To confirm whether the iron-deficient condition directly induces the reduction in PRMT1 and PRMT3 expressions and ADMA formation, we examined ADMA formation in cells treated with DFO or DFX in the presence or absence of holo-transferrin. After treating with DFO or DFX for 16 h, cells were incubated with/without holo-transferrin for 10 h and cell lysates were subjected to immunoblotting. Thus, protein methylation decreased after treatment with DFO or DFX alone, whereas the incubation of DFO- or DFX-treated cells with holo-transferrin restored the levels of ADMA formation (Figure 3A and B). The incubation of DFO- or DFX-treated cells with holo-transferrin also increased the protein expressions of PRMT1 and PRMT3 compared to those in cells treated with DFO or DFX alone. These observations suggested that iron directly affects ADMA formation by changing PRMT expression.

3.4. Dietary iron deficiency also downregulated PRMTs and decreased ADMA formation in rats

Next, we examined whether dietary iron deficiency affects PRMT expression and protein methylation in vivo using rats. There were no significant differences in food intake, final body weight, and liver weight (Supplemental data 1). Hemoglobin concentration and iron concentration in the liver were significantly lower in the ID group than in the CTL group (Figure 4A and B). In contrast, heart weight was significantly higher in the ID group than in the CTL group (Supplemental data 1). Therefore, these data suggested that iron-deficient anemia occurred in rat fed the iron-deficient diet. To determine the effect of iron deficiency on gene and protein expressions of PRMTs in the liver, we extracted mRNA and protein from the rat liver tissue. As a result, PRMT1 and PRMT3 gene and protein expressions in the ID group were lower than those in the CTL group (Figure 4C and D). Subsequently, we examined whether iron deficiency affects ADMA formation in the liver. Although we observed a robust increase in total ADMA content in the CTL group, ADMA formation was not detected in the ID group (Figure 4D).

4. Discussion

Iron is an essential element for cellular responses [1, 2, 3]. Our previous study using microarray analysis indicated that the downregulation of senescence marker protein 30 by DFO drives cell senescence [13]. In the same study, we also observed that PRMTs, which are involved in post-translational protein methylation, were downregulated under iron-deficient conditions (unpublished data). However, limited information is available on the molecular mechanisms underlying the association between PRMTs and iron-deficient conditions. In the present study, we demonstrated for the first time that both gene and protein expressions of PRMTs are significantly decreased under iron-deficient conditions. These results suggest that iron-deficiency-induced downregulation of PRMTs might affect protein methylation in the liver.

Several studies showed that both increases and decreases of PRMTs and protein methylation were observed in various oxidative stress conditions such as H_2O_2, lysophosphatidylcholine, and nitric oxide [19, 20, 21, 22, 23, 24]. Previously, Bautisa et al. reported that iron-overload treatment increased ROS production by decreasing the levels of both PRMT1 and catalase [25]. However, downregulation of PRMTs and protein methylation in iron-deficient condition has not yet been clearly understood. Interestingly, our group reported that iron deficiency induced ROS production [26]. To the best of our knowledge, this is the novel discovery that showed that protein methylation and expression of PRMTs during iron-deficient conditions are negatively regulated in vitro and in vivo. Thus, it is possible that iron-deficient conditions decreased the levels of PRMT1 and PRMT3 by increasing ROS production, leading to decreased levels of protein methylation.

In general, iron deficiency increases transferrin receptor 2 (Tfr2) expression, resulting in recovery of intracellular iron homeostasis [27]. Physiologic levels of holo-transferrin increases Tfr2 and reflects the changes in the saturation state of iron binding by transferrin [28]. Interestingly, the effect of iron-deficient conditions by DFO treatment was reversed by iron-saturated transferrin (holo-transferrin) but not by iron-free transferrin (apo-transferrin) [29]. Therefore, incubation with holo-transferrin can increase the ferrous iron concentration. In the present study, we observed that holo-transferrin treatment attenuated the
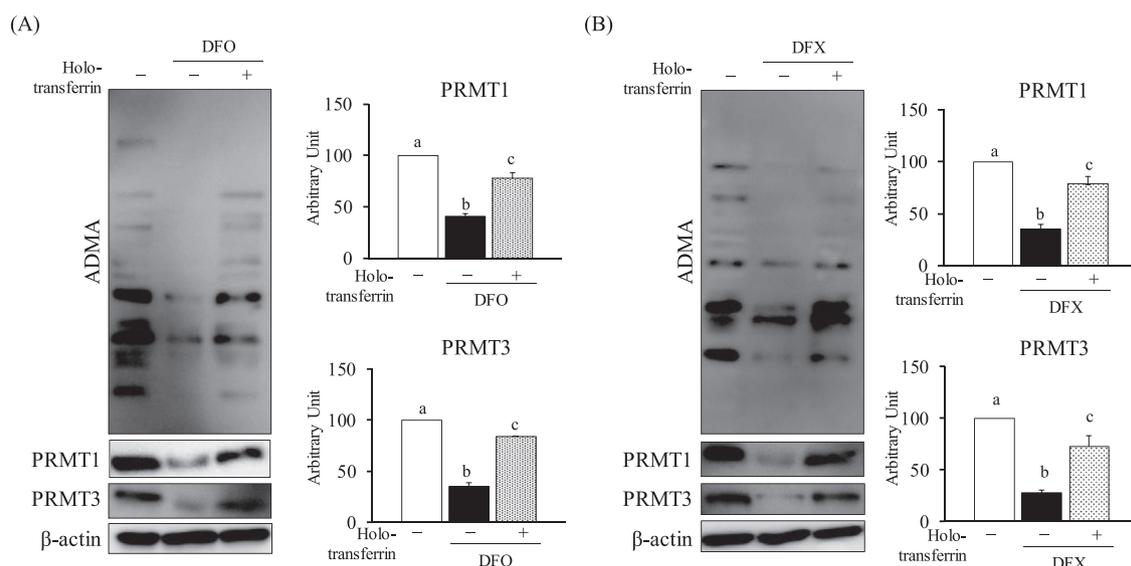
Figure 3. Holo-transferrin attenuated down-regulation of protein methylation in DFO or DFX treated cells. FAO cells were incubated with DFO or DFX for 16 h. After incubation with (A) DFO or (B) DFX for this period, 50 μM of human holo-transferrin was added for 10 h as an iron source. At the incubation, cells were lysed and the lysates were immunoblotted with the indicated antibodies. β-Actin was used as an internal control. Protein levels were normalized to β-actin. Data are shown as the means ± S.E., from at least 3 independent experiments. Multiple comparisons were performed using Tukey's test after one-way ANOVA. Unmatching letters denote significant differences, $p < 0.05$. The full uncropped images are available as supplementary material.
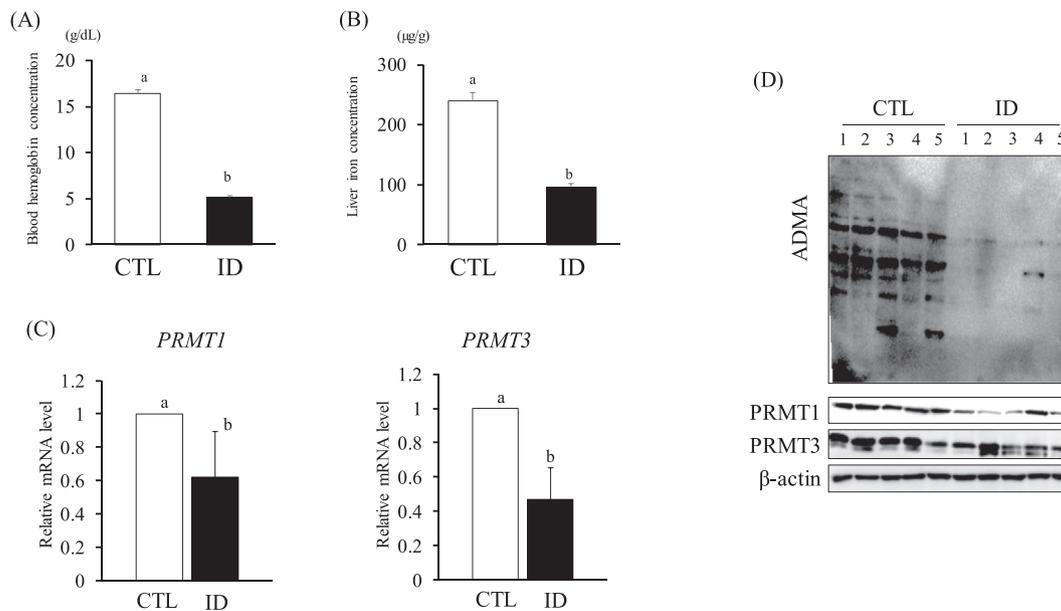
Figure 4. Dietary iron-deficiency downregulated PRMTs expression and decreased protein methylation in the liver. (A) Blood hemoglobin concentration and (B) iron concentration in liver. (C) RNA was extracted from liver tissue, and cDNA was synthesized. Then, the gene expression levels of *PRMT1* and *PRMT3* mRNA were quantified by real time PCR, with *GAPDH* serving as the internal standard. (D) After liver tissues were homogenized with 4-fold of homogenization buffer, western blot analyses were performed to detect the indicated antibodies. β-Actin was used as internal control. Data are shown as the means ± S.E. from 6 rats. Unmatching letters denote significant differences, $p < 0.05$ using Student's t-test. The full uncropped images are available as supplementary material.

downregulation of PRMT1 and PRMT3 expression in iron-deficient conditions. Interestingly, we found that the supplementation of holo-transferrin also obviously increased protein methylation under iron-deficient conditions. Furthermore, we observed dietary iron-deficient rats showed decreased PRMT1 and PRMT3 expression and protein methylation compared to the control rats. In fact, hepatic TfR2 levels in iron-deficient rats were reduced compared to those in rats fed the control diet with normal iron levels [30, 31]. Thus, we proposed that low iron status reflects protein methylation levels *in vivo* as well as *in vitro*. This result may imply that iron content can regulate protein methylation by increasing PRMT expression.

Protein methylation and PRMT1 protein levels decrease in young WI-38 human diploid fibroblasts compared to replicative senescent fibroblasts [20]. In addition, several researchers have reported the fundamental roles of protein methylation in aging [32, 33]. Our data suggested that iron-deficient conditions could be linked with protein methylation by downregulation of PRMTs and cellular senescence. Therefore, we proposed that iron deficiency-induced downregulation of PRMTs and protein methylation not only maintained iron homeostasis but also promoted cell senescence. However, in the present study, the investigation on the relationship between protein methylation and the aging process in iron-deficient conditions was insufficient. Further *in vivo* studies are required in the long term.

Finally, we investigated the effects of iron-deficient conditions on protein methylation via PRMTs. To our knowledge, this is the first study to report that DFO and DFX decreased protein methylation by down-regulating the expression of PRMT1 and PRMT3 *in vitro* and *in vivo*. In conclusion, these results suggested that protein methylation and PRMTs might correlate iron status with cellular homeostasis including cell senescence.

Declarations

Author contribution statement

H. Inoue: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

M. Uehara: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

N. Hanawa: Performed the experiments; Analyzed and interpreted the data.

Y. Aizawa: Performed the experiments.

S. Katsumata: Analyzed and interpreted the data.

R. Tsuboi-Katsumata, M. Tanaka, N. Takahashi: Contributed reagents, materials, analysis tools or data.

Competing interest statement

The authors declare no conflict of interest.

Additional information

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