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ORIGINAL ARTICLE

Reduction of a marker of oxidative stress with enhancement of iron utilization by erythropoiesis activation following epoetin beta pegol administration in iron-loaded db/db mice

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Abstract Iron, an essential element for various biological processes, can induce oxidative stress. We hypothesized that iron utilization for erythropoiesis, stimulated by epoetin beta pegol (C.E.R.A.), a long-acting erythropoiesis-stimulating agent, contributes to the reduction of iron-induced oxidative stress. We first investigated the sensitivity of several biomarkers to detect oxidative stress in mice by altering the amount of total body iron; we then investigated whether C.E.R.A. ameliorated oxidative stress through enhanced iron utilization. We treated db/db mice with intravenous iron-dextran and evaluated several biomarkers of iron-induced oxidative stress. In mice loaded with 5 mg/head iron, hepatic iron content was elevated and the oxidative stress marker d-ROMs (serum derivatives of reactive oxygen metabolites) was increased, whereas urinary 8-hydroxy-2′-deoxyguanosine and serum malondialdehyde were not, indicating that d-ROMs is a sensitive marker of iron-induced oxidative stress. To investigate whether C.E.R.A. ameliorated oxidative stress, db/db mice were intravenously administered iron-dextran or dextran only, followed by C.E.R.A. Hemoglobin level increased, while hepatic iron content decreased after C.E.R.A. treatment. Serum d-ROMs decreased after C.E.R.A. treatment in the iron-dextran-treated group. Our results suggest that C.E.R.A. promotes iron utilization for erythropoiesis through mobilization of hepatic iron storage, leading to a decrease in serum oxidative stress markers in iron-loaded db/db mice.

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Introduction

Iron is an essential element for mammals as it is a component of many key redox enzymes and oxygen storage and transporting proteins such as myoglobin and hemoglobin (Hb) [1]. On the other hand, iron has potential to induce oxidative stress. Excess iron causes oxidative stress via production of reactive oxygen species (ROS) such as hydroxyl radicals through the Fenton and Haber–Weiss reactions, which cause tissue damage via injuries to DNA, proteins, lipids, and carbohydrates [2]. Therefore, iron is strictly regulated [3]. Iron trafficking is negatively regulated by hepcidin, a 25-amino acid peptide hormone produced mainly in the liver, which regulates intestinal iron absorption and iron release from hepatic stores by causing degradation of the iron transporter ferroportin in enterocytes and reticuloendothelial macrophages [1, 4].

ROS are a family of molecules including oxygen and its derivatives that are produced in aerobic cells. Excessive ROS production, outstripping endogenous antioxidant defense mechanisms, is commonly referred to as oxidative stress [5]. Biomarkers of oxidative stress can be classified as molecules that are modified by interactions with ROS in the microenvironment and molecules of the antioxidant system that change in response to increased oxidative stress [6]. Measuring biomarkers in serum, plasma, or urine is useful in an outpatient setting for evaluating oxidative stress levels which can provide important insights into the pathophysiology of oxidative stress-related diseases. Oxidative stress has been

implicated as a crucial factor in the pathogenesis and development of a variety of chronic and degenerative diseases, including aging and cancer [5–7], and in a variety of cardiovascular diseases, such as hypertension, arterial sclerosis, ischemic cardiac disease, diabetes mellitus, and heart failure [6, 8, 9].

Erythropoiesis and iron metabolism are inextricably linked. During erythropoiesis, a sufficient supply of iron is required to promote erythroblast maturation [10]. Developing immature erythroblasts have an extremely high iron requirement, especially during Hb synthesis [11]. Epoetin beta pegol (continuous erythropoietin receptor activator, C.E.R.A.) is a novel long-acting erythropoiesis-stimulating agent (ESA) that is approved for the correction and maintenance of Hb levels in patients with renal anemia with up to once-monthly administration. In a previous study, we showed that C.E.R.A. promoted utilization of iron for erythropoiesis through intensive suppression of serum hepcidin levels. Although C.E.R.A. is expected to have a strong effect on enhancing iron utilization, the effects of C.E.R.A. on oxidative stress through mobilization of iron storage are not fully understood.

We hypothesized that utilization of iron for erythropoiesis would contribute to the reduction of iron-induced oxidative stress. In the present study, to reveal the relationship between oxidative stress and enhancement of iron utilization by erythropoiesis activation, we first examined the sensitivity of several oxidative stress markers to detect iron-induced oxidative stress, and then using the most sensitive marker, we investigated whether C.E.R.A. could ameliorate oxidative stress with enhancement of iron utilization in db/db mice.

Materials and methods

Chemicals

Iron-dextran, dextran, Dulbecco's phosphate buffered saline (PBS), Iscove's Modified Dulbecco's Media (IMDM), polyoxyethylene sorbitan monooleate (Tween 80) were purchased from Sigma-Aldrich (St. Louis, MO, USA). C.E.R.A. was produced by Chugai Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Bovogen Biologicals (East Keilor, Australia).

Animals

Six- to 10-week-old male BKS.Cg-Dock7^{m}+/+Lepr^{db}/J (db/db) mice were obtained from Charles River Laboratories Japan (Kanagawa, Japan). The mice were acclimatized for at least 4 days in our animal facility before use. Mice were fed rodent chow and water ad libitum. All animal experiments were performed in accordance with the *Guidelines for the Care and Use of Laboratory Animals* at Chugai Pharmaceutical Co., Ltd.

Animal treatment

C.E.R.A., iron-dextran, and dextran were diluted to appropriate concentrations in a phosphate-buffered vehicle (PBS containing 0.02 % Tween 80).

To evaluate iron-induced oxidative stress markers, mice were administered intravenous iron-dextran or dextran once a day for 5 days to a total iron loading of 0, 5, or 50 mg/head iron. Nine days after the first injection, 8 mice from each group were euthanized by exsanguination under anesthesia with isoflurane and specimens were collected.

To investigate the effects of C.E.R.A. on oxidative stress markers, mice were administered a single intravenous injection of iron-dextran (0.5 mg/head iron) or dextran to change their iron loading. Five days later, mice were intravenously treated with a single injection of 10 μg/kg C.E.R.A. or vehicle. At 1 or 8 days after treatment with C.E.R.A. or vehicle, mice (Day 1: 3 in each group; Day 8: 4 or 5 in the dextran group, 8 in the iron-dextran group) were euthanized by exsanguination under anesthesia with isoflurane and specimens were collected.

To decrease total body iron by restricting dietary iron absorption, mice were acclimatized to a control diet containing 103 ppm iron (Research Diets, New Brunswick, NJ, USA) and distilled water (Wako Pure Chemical Industries, Tokyo, Japan) for 5 days. All mice were injected with a single intravenous injection of iron-dextran (0.5 mg/head iron), and the control diet was then switched to an iron-deficient diet containing 3 ppm iron in the iron-deficient group. The control group was continuously fed the control diet. Fourteen days after the diet was switched, 3 or 4 mice from each group were euthanized by exsanguination under anesthesia with isoflurane and specimens were collected.

Specimen collection

Blood was collected into Minicollect ethylenediaminetetraacetic acid (EDTA) tubes and Minicollect serum tubes (Greiner Bio-One, Kremsmünster, Austria). Blood samples were analyzed immediately to determine hematological parameters, and serum was isolated according to the manufacturer's instructions and stored at −80 °C until use for assays. Urine was collected and stored at −80 °C until use for assays. The livers were collected from the mice in each group and divided into two portions. The livers in one

portion were individually weighed and stored at −80 °C until their use for assays to measure iron content. The livers in the other portion were fixed in 10 % neutral buffered formalin for histological analysis. For flow cytometry analysis, bone marrow cells were collected from the femur by flushing out cells with IMDM containing 2 % FBS. Bone marrow cells were stored on ice. For mRNA extraction, bone marrow cells were collected from the femur by flushing out cells with RLT buffer (Qiagen, Valencia, CA, USA) containing 1 % 2-mercaptoethanol (Wako Pure Chemical Industries) and were immediately frozen in liquid nitrogen.

Measurement of hematological and iron indices

Hematological parameters were measured with an automated hematology analyzer (XT-2000iV; Sysmex, Hyogo, Japan). Serum iron level and UIBC levels were measured with an automatic biochemistry analyzer (TBA-2000FR; Toshiba Medical Systems, Tochigi, Japan). Concentrations of serum hepcidin were measured by sensitive liquid chromatography/electrospray ionization tandem mass spectrometry using an AB SCIEX QTRAP 5500 system (AB Sciex, Foster City, CA, USA) equipped with a Prominence UFLC^{XR} system (Shimadzu Corporation, Kyoto, Japan) as previously reported [12, 13]. Hepatic iron content was determined by inductively coupled plasma-optical emission spectrometry analysis.

Analysis of the population of TER119^{positive}/CD71^{high} cells in bone marrow cells by flow cytometry

Cells collected from the femur were prepared by washing with PBS containing 2 % FBS. Cells (1×10^6) were incubated with anti-CD16/CD32 antibody, followed by staining with fluorescein isothiocyanate (FITC)-conjugated anti-TER119 monoclonal antibody and phycoerythrin (PE)-conjugated anti-CD71 monoclonal antibody. Cells were again washed with PBS containing 2 % FBS, and then stained with 7-amino-actinomycin D (7-AAD) to exclude nonviable cells in flow cytometric analysis. All antibodies and 7-AAD were purchased from BD Pharmingen (San Diego, CA, USA). Analysis of fluorescently labeled cells was performed using FACS Aria II (BD BioScience, Tokyo, Japan).

Measurement of oxidative stress markers

Serum levels of malondialdehyde (MDA) and urinary levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) were determined using ELISA kits (Nikken SEIL, Shizuoka, Japan). Creatinine was measured using a creatinine parameter assay kit (R&D Systems, Minneapolis, MN, USA). Serum levels of hydroperoxide, a marker for oxidative stress, were determined using the derivatives of reactive oxygen metabolites (d-ROMs) test according to the manufacturer's instructions (FREE Carpe Diem; Diacron International, Grosseto, Italy). This test is based on the ability of transition metal ions to catalyze the breakdown of hydroperoxide, generating new radical species such as hydroxyperoxyl and alkoxyl. These free radicals are trapped by an alkylamine chromogen which changes color when oxidized by free radicals and can be detected by absorbance at 505 nm. A sample of serum (20 µL) was mixed with an acid buffer solution (pH 4.8, Reagent R1 of the kit) in a cuvette, and was then supplemented with 20 µL of the chromogen (Reagent R2 of the kit). Samples were mixed and were then incubated and read for optical density by FREE Carpe Diem. The unit used in the d-ROMs test is 1 U.CARR, which corresponds to 0.8 mg/L of hydrogen peroxide.

Histopathology

Sections (4 µm thick) were prepared from paraffin-embedded formalin-fixed liver. Hepatic hemosiderin deposition was assessed by Berlin blue staining as previously reported [14].

Quantification of erythroferrone mRNA levels

Total RNA was isolated from bone marrow cells by using an RNeasy Mini Kit (Qiagen) and Sepasol-RNA I Super G reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturers' instructions. Total RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Indianapolis, IN, USA) as directed by the manufacturer. The primer pairs were targeted to the genes coding for mouse *erythroferrone* (NM_173395.2) and *β-actin* (NM_007393.5). Sequences of primers were as follows: *erythroferrone* primer, AGC GAG CTC TTC ACC ATC TC and TGT CCA AGA AGA CAG AAG TGT AGT G; *β-actin* primer, CCA CTG CCG CAT CCT CT and CCA CAG GAT TCC ATA CCC AAG AAG. Afterwards, quantitative PCR was performed under identical conditions and expression of *erythroferrone* transcripts was normalized to that of the reference gene *β-actin*. Real-time PCR was carried out using the LightCycler 480 (Roche Diagnostics). LightCycler 480 Probes Master and probe #96 from Universal ProbeLibrary were used for *erythroferrone*, and LightCycler 480 SYBR Green I Master was used for *β-actin*, according to the manufacturer's instructions (Roche Diagnostics).

Statistical analysis

Statistical analysis was performed by Tukey's test, Steel–Dwass test or Student's *t* test using JMP software (SAS Institute, Cary, NC, USA). A P value of <0.05 was considered statistically significant. Data are represented as mean and SD.

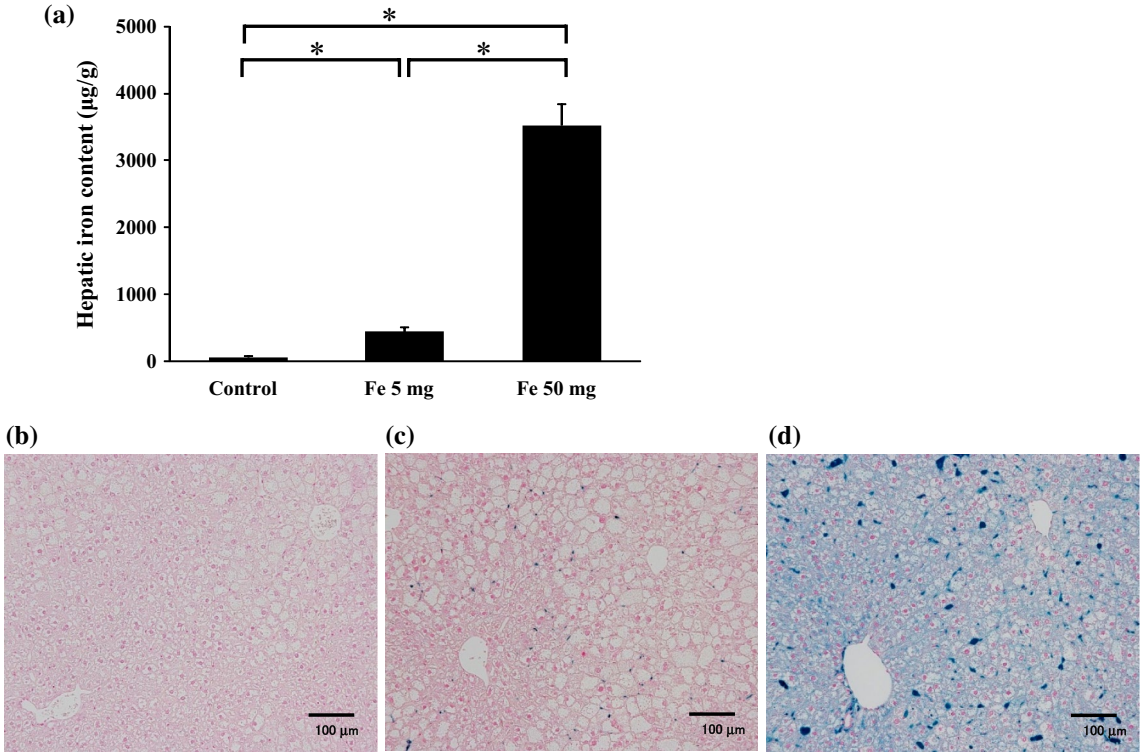

Fig. 1 The status of iron storage in iron-loaded mice 9 days after the first injection of iron-dextran or dextran. **a** Hepatic iron content in iron-loaded mice. Results are the mean + SD for 8 mice in each group. Statistical significances were analyzed by Tukey's test; $^*P < 0.05$. **b–d** Histopathological analysis of hepatic hemosiderin deposition in iron-loaded mice. Representative images of hepatic hemosiderin deposition in mice treated with iron-dextran or dextran: **b** control group, **c** iron 5 mg group, and **d** iron 50 mg group

Results

Iron-loaded mice showed iron dose-dependent increase in iron accumulation in liver

We analyzed changes in iron metabolism and oxidative stress in db/db mice by altering their iron status. We treated db/db mice with intravenous iron-dextran or dextran for 5 days to change their iron storage status (total amount of iron administered: 0, 5, or 50 mg/head).

To confirm accumulation of iron in iron storage organs and to investigate iron metabolism in the iron-loaded mice, we measured hepatic iron content, hepatic hemosiderin deposition as assessed by Berlin blue staining, serum hepcidin levels, serum iron levels, and hematological parameters. An iron dose-dependent increase in hepatic iron content was observed (Fig. 1a). Consistent with hepatic iron content, iron dose-dependent hepatic hemosiderin deposition was detected on Kupffer cells and macrophages 9 days after the first injection (Fig. 1b–d). Compared to the control group, iron-loaded mice exhibited a dose-dependent increase in hepcidin levels and serum iron levels, and a dose-dependent decrease in UIBC levels, whereas no difference was observed in reticulocyte counts, Hb levels, and RBC counts (Table 1).

Table 1 Hematological and iron metabolism-related parameters 9 days after the first injection of iron-dextran or dextran

Parameters	Control	Fe 5 mg	Fe 50 mg
Reticulocytes ($\times 10^2$/μL)	2943 ± 540	3109 ± 715	2995 ± 479
Red blood cells ($\times 10^4$/μL)	999 ± 47	1019 ± 93	1000 ± 52
Hb (g/dL)	14.74 ± 0.85	15.19 ± 1.46	14.45 ± 0.82
Serum iron (μg/dL)	219.8 ± 15.7	320.4 ± 25.9*	469.9 ± 42.3*,#
UIBC (μg/dL)	219.8 ± 26.9	80.0 ± 35.2*	8.3 ± 2.5*,#
Hepcidin (ng/mL)	108.2 ± 23.3	194.8 ± 32.3*	245.5 ± 50.7*,#

Results are the mean ± SD for 8 mice in each group. In UIBC, statistical significances were analyzed by Steel–Dwass test and, in other parameters, statistical significances were analyzed by Tukey's test

* $P < 0.05$, control group *vs.* Fe 5 mg or Fe 50 mg group. # $P < 0.05$, Fe 5 mg group *vs.* Fe 50 mg group

Iron-loaded mice showed iron dose-dependent increase in oxidative stress marker d-ROMs

To determine a sensitive marker reflecting iron-induced oxidative stress, we investigated d-ROMs (a novel marker) as well as levels of 8-OHdG and MDA in iron-loaded mice.

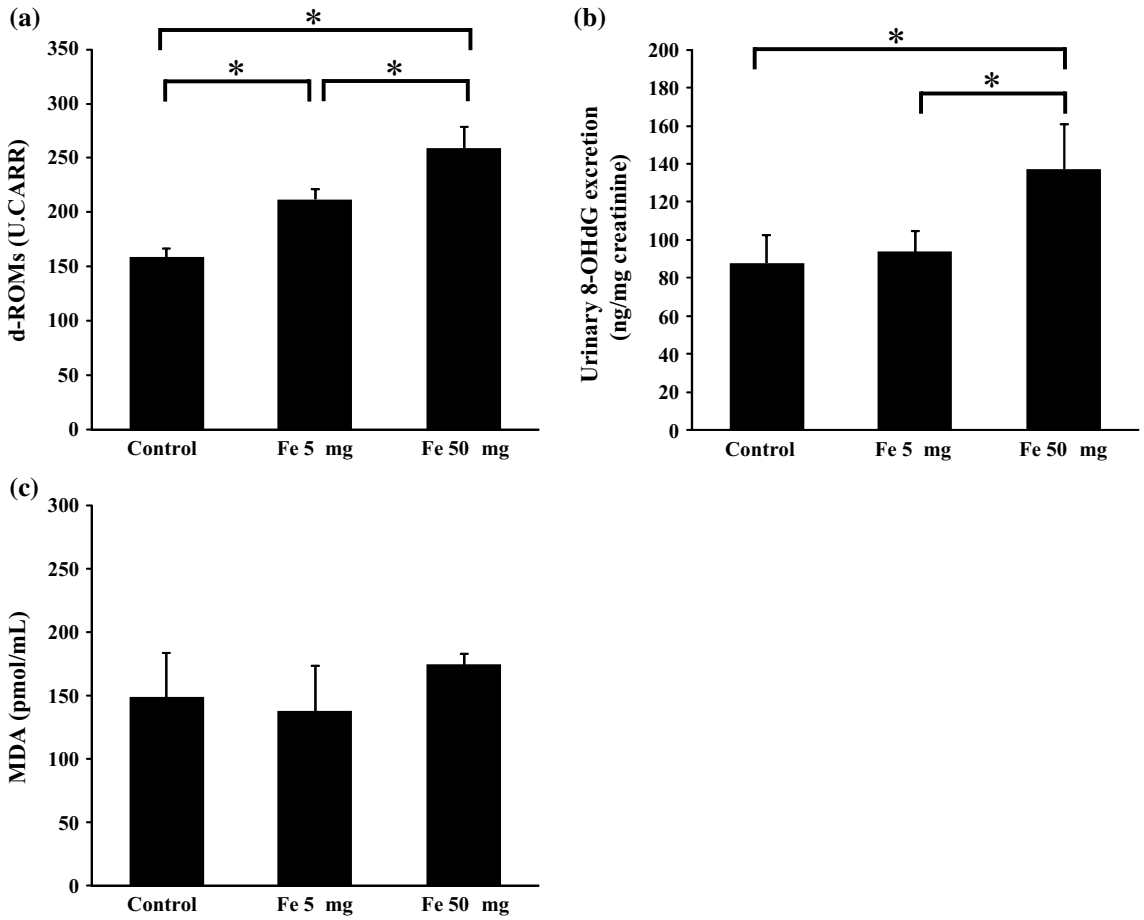

Fig. 2 Oxidative stress markers in iron-loaded and control mice 9 days after the first injection of iron-dextran or dextran. **a** Serum d-ROMs levels, **b** urinary 8-OHdG levels, and **c** serum MDA levels. Results are the mean + SD for 8 mice in each group. Statistical significances were analyzed by Tukey's test; $^{*}P < 0.05$

Iron-loaded mice showed a dose-dependent increase in serum d-ROMs 9 days after the first injection (Fig. 2a). Urinary 8-OHdG level was significantly higher in the 50 mg group than in the control group or 5 mg group, whereas no change was observed in serum MDA levels (Fig. 2b, c).

Iron utilization was enhanced with increased erythropoietic activity after C.E.R.A. administration to mice

We investigated whether C.E.R.A. could ameliorate oxidative stress with enhancement of iron utilization in mice. We intravenously treated db/db mice with iron-dextran [0.5 mg/head iron; Fe(+) group] or dextran [Fe(−) group] to change their iron storage status. Five days later, C.E.R.A. (10 μg/kg) or vehicle was administered intravenously to both groups (Day 0).

On Day 0, to confirm iron accumulation in iron storage organs and the increase of oxidative stress 5 days after iron loading, we measured iron indices (serum hepcidin and iron levels), hepatic iron content, and d-ROMs. Serum hepcidin level and hepatic iron content were significantly higher in the Fe(+) group than in the Fe(−) group, whereas no difference was observed in serum iron level (Fig. 3a–c). Serum d-ROMs was higher in the Fe(+) group than in the Fe(−) group (Fig. 3d).

Because accumulation of iron in the liver and an increase in oxidative stress marker were confirmed in the Fe(+) group on Day 0, we consequently intravenously injected both groups with C.E.R.A. (10 μg/kg) or vehicle. On Day 1 and on Day 8, we investigated erythropoietic parameters and iron metabolism-related parameters to investigate the enhancement of iron utilization through erythropoiesis in C.E.R.A.-treated mice. To examine the differentiation of bone marrow cells after erythropoiesis stimulation by C.E.R.A. treatment, TER-119^{positive}/CD71^{high} cells were analyzed by flow cytometry (Table 2; Fig. 4a). On Day 1, we could not detect

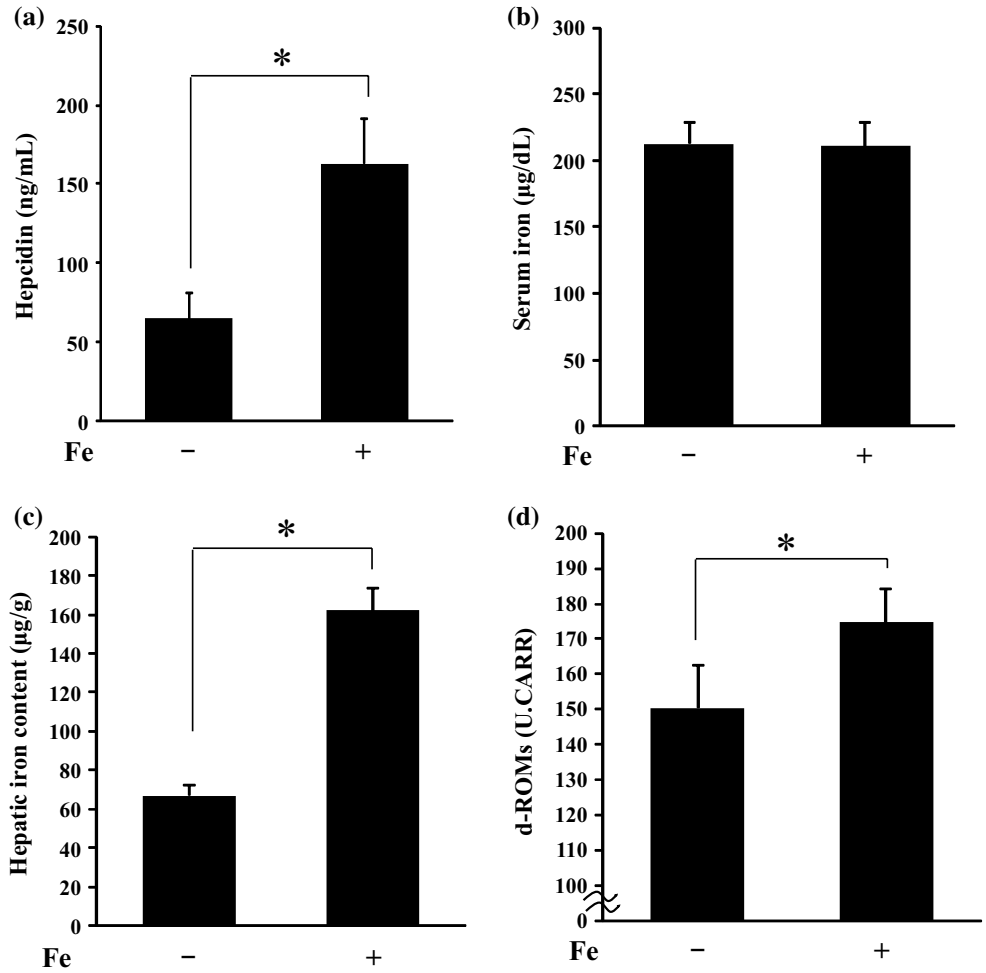

Fig. 3 Iron indices and oxidative stress marker levels 5 days after injection of iron-dextran or dextran (Day 0). **a** Serum hepcidin levels, **b** serum iron levels, **c** hepatic iron content, and **d** serum d-ROMs levels 5 days after injection of iron-dextran or dextran (Day 0). Results are mean + SD for 4 mice in the Fe(−) group and 8 mice in the Fe(+) group. Statistical significances were analyzed by Student's *t* test; **P* < 0.05 *vs.* vehicle group

Table 2 Hematological and iron metabolism-related parameters 1 day after the injection of C.E.R.A. or vehicle (Day 1)

Parameters	Fe(–) + vehicle	Fe(–) + C.E.R.A.	Fe(+) + vehicle	Fe(+) + C.E.R.A.
Erythroferrone/β-actin ratio ($\times 10^{-3}$)	N.D.	1.12 ± 0.16	N.D.	0.87 ± 0.20
Hepcidin (ng/mL)	50.8 ± 7.06	24.4 ± 9.56*	77.1 ± 14.1	41.8 ± 8.71*
TER119^{positive}/CD71^{high} erythroblasts (%)	34.77 ± 5.95	33.50 ± 2.29	35.27 ± 1.93	32.87 ± 3.10
Hepatic iron content (μg/g)	61.56 ± 13.04	43.14 ± 3.17	131.62 ± 4.32	147.74 ± 16.08

ND not detected

Results are the mean ± SD for 3 mice in the each group. Statistical significances were analyzed by Student's *t* test; * *P* < 0.05 *vs.* vehicle group

erythroferrone mRNA expression levels normalized by *β-actin* after vehicle treatment in either Fe(–) or Fe(+) groups; in contrast, *erythroferrone* mRNA expression levels were higher after C.E.R.A. treatment in both the Fe(–) and Fe(+) groups (Table 2). Hepcidin levels were significantly lower after C.E.R.A. treatment in both the Fe(–) and Fe(+) groups (Table 2). No differences were observed in the population of TER119^{positive}/CD71^{high} cells and hepatic iron content (Table 2). On Day 8, the population of TER119^{positive}/CD71^{high} cells was significantly higher in C.E.R.A.-treated mice than in vehicle-treated mice in both the Fe(−) and Fe(+)

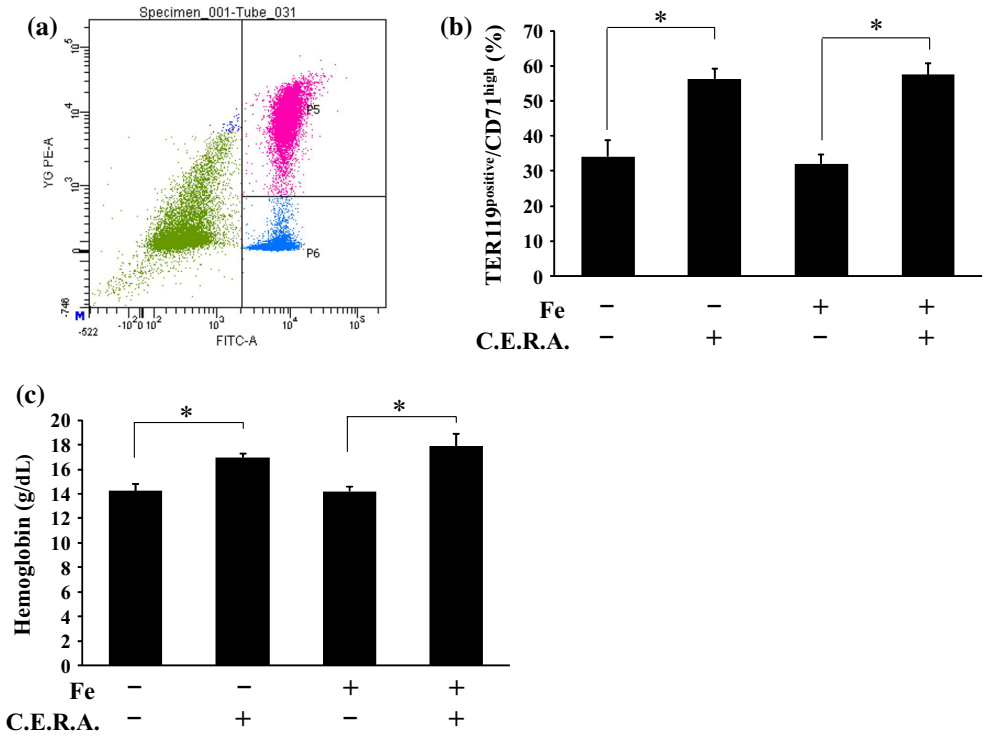

Fig. 4 Population of TER119^{positive}/CD71^{high} cells in bone marrow cells and Hb levels 8 days after the injection of C.E.R.A. or vehicle (Day 8). **a** Representative image of the population of TER119^{positive}/CD71^{high} cells in bone marrow cells from the vehicle-treated Fe(−) group. **b** Average percentages of the populations of TER119^{positive}/CD71^{high} cells in bone marrow cells. **c** Hb levels 8 days after the injection of C.E.R.A. or vehicle. Results are the mean + SD for 5 mice in the Fe(−) group and 8 mice in the Fe(+) group. Statistical significances were analyzed by Student's *t* test; **P* < 0.05 *vs.* vehicle group

Table 3 Hematological and iron metabolism-related parameters 8 days after the injection of C.E.R.A. or vehicle (Day 8)

Parameters	Fe(−) + vehicle	Fe(−) + C.E.R.A.	Fe(+) + vehicle	Fe(+) + C.E.R.A.
Reticulocytes (×10^2/μL)	2152 ± 348	22,952 ± 1984*	2014 ± 265	22,472 ± 2315*
Red blood cells (×10^4/μL)	949 ± 40	1109 ± 36*	944 ± 42	1149 ± 65*
Serum iron (μg/dL)	194.8 ± 14.5	71.8 ± 15.3*	169.6 ± 19.7	74.3 ± 24.3*
Hepcidin (ng/mL)	76.2 ± 14.5	36.8 ± 3.78*	129.2 ± 18.6	44.4 ± 7.12*

Results are the mean ± SD for 5 mice in the Fe(−) group and 8 mice in the Fe(+) group. Statistical significances were analyzed by Student's *t* test; * *P* < 0.05 *vs.* vehicle group

groups (Fig. 4b). Hb level was significantly higher after C.E.R.A. treatment in both the Fe(−) and Fe(+) groups (Fig. 4c). Reticulocyte counts and RBC counts were significantly higher after C.E.R.A. treatment in both the Fe(−) and Fe(+) groups (Table 3), and serum hepcidin level and serum iron level were significantly lower in C.E.R.A.-treated mice (Table 3). We could not detect *erythroferrone* mRNA levels in any of the groups on Day 8 (data not shown).

To evaluate the iron status in iron storage organs, we measured hepatic iron content and evaluated hemosiderin deposition in the liver by Berlin blue staining. Hepatic iron content was lower in C.E.R.A.-treated mice than in vehicle-treated mice in both the Fe(−) and Fe(+) groups (Fig. 5a). Hepatic hemosiderin deposition on Kupffer cells and macrophages was less prominent in the C.E.R.A.-treated mice than in the vehicle-treated mice in the Fe(+) group (Fig. 5b, c).

Suppression of oxidative stress marker d-ROMs was observed after C.E.R.A. administration in iron-loaded mice

To evaluate the impact that iron utilization enhanced through erythropoiesis had on oxidative stress, we measured the oxidative stress marker d-ROMs. Serum d-ROMs

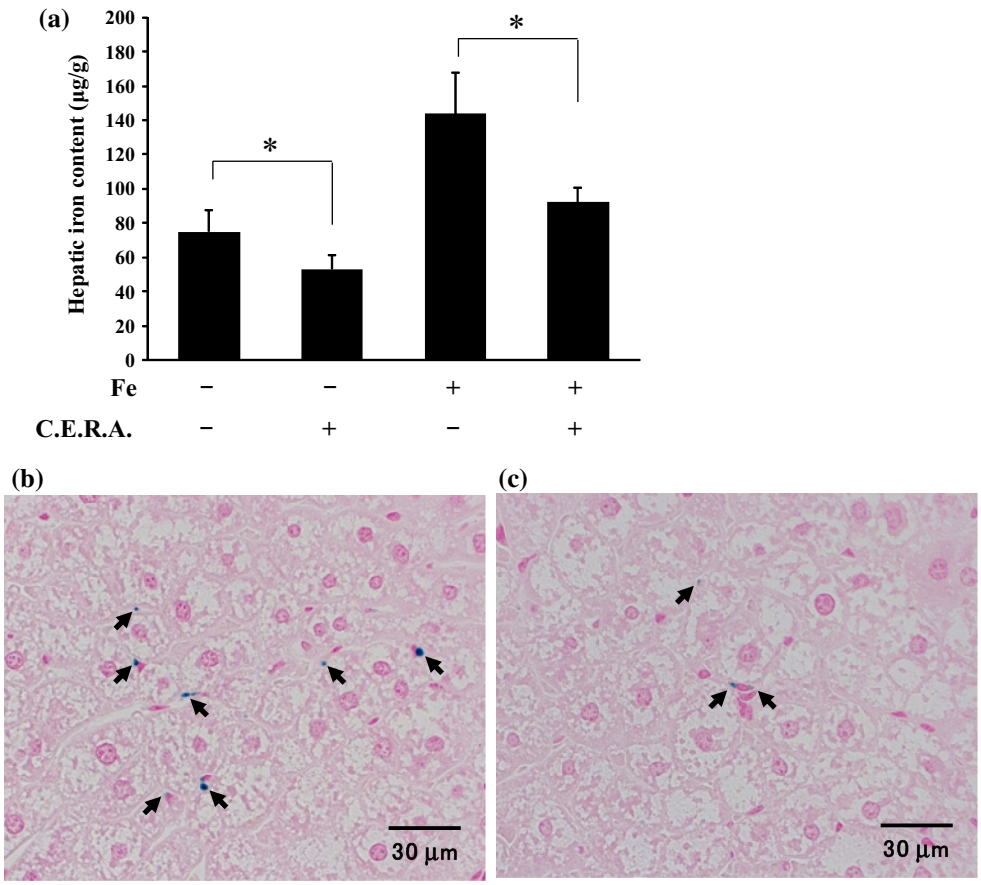

Fig. 5 Iron storage status 8 days after the injection of C.E.R.A. or vehicle (Day 8). **a** Hepatic iron content 8 days after the injection of C.E.R.A. or vehicle. Results are the mean + SD for 5 mice in the Fe(−) group and 8 mice in the Fe(+) group. Statistical significances were analyzed by Student's *t* test; $*P < 0.05$ *vs.* vehicle group. **b**, **c** Histopathological analysis of hepatic hemosiderin deposition 8 days after the injection of C.E.R.A. or vehicle in mice in the Fe(+) group. Representative images of hepatic hemosiderin deposition in mice in **b** the vehicle-treated group and **c** the C.E.R.A.-treated group

was significantly lower in the C.E.R.A.-treated Fe(+) group than in the vehicle-treated Fe(+) group on Day 8 (Fig. 6). On the other hand, although d-ROMs also decreased in the C.E.R.A.-treated Fe(−) group as compared to the vehicle-treated Fe(−) group on Day 8, this decrease was not significant (Fig. 6).

Suppression of oxidative stress marker d-ROMs was observed in iron-loaded mice fed an iron-deficient diet

To confirm that the decrease in d-ROMs observed in the C.E.R.A.-treated Fe(+) group occurred via iron, we evaluated hepatic iron content and d-ROMs in mice that were first loaded with 0.5 mg/head iron and were then fed an iron-deficient diet to decrease total body iron by restricting dietary iron absorption. Hepatic iron content was significantly lower in the iron-deficient diet group than in the control diet group (Fig. 7a). Serum d-ROMs was significantly lower in the iron-deficient diet group than in the control diet group (Fig. 7b). No significant differences were observed in reticulocyte counts or RBC counts (Table 4). Serum iron levels and hepcidin levels tended to be lower in the iron-deficient diet group than in the control diet group; however, there were no statistical differences between them (Table 4).

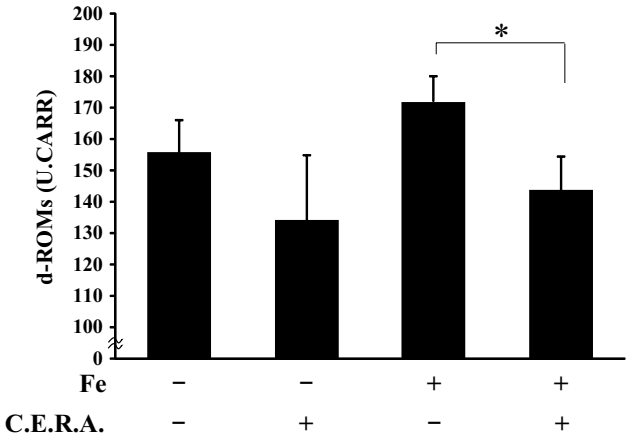

Fig. 6 Serum d-ROMs levels 8 days after the injection of C.E.R.A. or vehicle (Day 8). Results are the mean + SD for 5 mice in the Fe(−) group and 8 mice in the Fe(+) group. Statistical significances were analyzed by Student's *t* test; $*P < 0.05$ *vs.* vehicle group

Discussion

In the present study, we demonstrated that d-ROMs was a sensitive marker of iron-induced oxidative stress, that

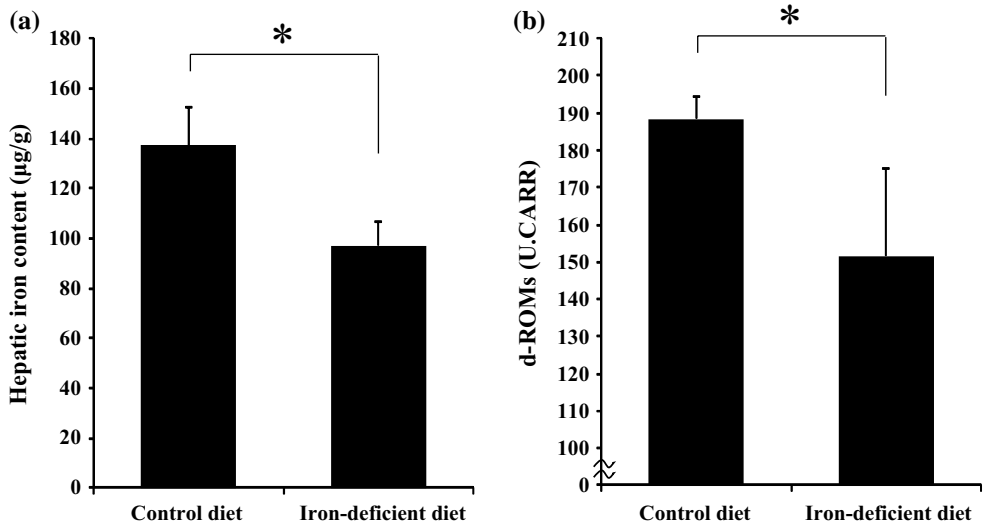

Fig. 7 Hepatic iron content and serum d-ROMs levels 14 days after mice were fed an iron-deficient diet or control diet. Results are the mean + SD for 4 mice in the control diet group and 3 mice in the iron-deficient diet group. Statistical significances were analyzed by Student's *t* test; **P* < 0.05 *vs.* control diet group

Table 4 Hematological and iron metabolism-related parameters in mice fed control or iron-deficient diet for 14 days

Parameters	Control diet	Iron-deficient diet
Reticulocytes ($\times 10^2$/μL)	2805 ± 684	3139 ± 537
Red blood cells ($\times 10^4$/μL)	1015 ± 40	1073 ± 89
Serum iron (μg/dL)	191.5 ± 36.0	142.7 ± 26.3
Hepcidin (ng/mL)	163.3 ± 30.2	119.0 ± 11.1

Results are the mean ± SD for 4 mice in the control diet group and 3 mice in the iron-deficient diet group. Statistical significances were analyzed by Student's *t* test; * $P < 0.05$ *vs*. control diet group

C.E.R.A. treatment promoted iron utilization for erythropoiesis through mobilization of iron from iron storage, and that consequently d-ROMs was decreased in db/db mice.

It is reported that iron metabolism dysfunction occurs in db/db mice, with greater serum iron levels and urinary iron excretion observed in db/db mice than in db/m control mice, and that dietary iron restriction exerts a preventive effect on the progression of diabetic nephropathy with the reduction of oxidative stress in db/db mice [15]. Moreover, it is also reported that oxidative stress is enhanced in response to hyperglycemia in diabetes mellitus [16]. Therefore, we chose to use this mouse strain to analyze changes in iron-induced oxidative stress and iron metabolism.

In the first part of our study, iron-loaded mice showed a dose-dependent increase in hepatic iron content and a dose-dependent increase in hemosiderin deposition in the liver (Fig. 1). Consistent with these phenomena, serum hepcidin levels and serum iron levels increased in response to iron loading (Table 1). These results showed that hepcidin was up-regulated in response to iron signaling, but uptake of iron into storage organs was not sufficient to cope with the supply of iron because iron overflow into serum was observed. This suggested that oxidative stress might be increased under these iron-overloaded conditions. We found d-ROMs to be a more sensitive marker of iron-induced oxidative stress than the well-established oxidative stress markers 8-OHdG and MDA; d-ROMs showed an iron dose-dependent increase that was consistent with the increased hepatic iron content and increased hemosiderin deposition in the liver (Figs. 1, 2). Several groups have reported that levels of a variety of oxidative stress markers, including 8-OHdG, increase in response to substantial iron overload; however, there are few reports of changes in oxidative stress markers at low levels of iron loading [17]. Our results demonstrated that d-ROMs can detect an increase in oxidative stress even under conditions of low-level iron loading (Figs. 2a, 3d). There was no difference between the iron-loaded groups and the control group in terms of reticulocyte counts, RBC counts, or Hb levels (Table 1), suggesting that excess iron was distributed in parts other than RBCs which are known to carry most of the iron in the body in the Hb; therefore, oxidative stress might originate from a place other than RBCs.

The test for d-ROMs, a newly established lipid peroxidation marker, is a simple assay that analyzes the total amount of hydroperoxides in serum via the Fenton reaction. d-ROMs has been reported as a novel biomarker of several diseases. Elevated oxidative stress as assessed by d-ROMs is a prognostic predictor of mortality in patients

with cardiovascular disease [7, 18]. d-ROMs has been reported as a novel plasma biomarker in the prediction of first atherothrombotic events [19]. In men who underwent a general health screening test, higher oxidative stress levels as assessed by d-ROMs were reported to be associated with lower estimated glomerular filtration rates (eGFR) and with increased albuminuria [20]. A profound imbalance between antioxidants and the production of ROS as assessed by d-ROMs was reported in end-stage renal disease patients, and the involvement of uremia was suggested [21]. It was reported that serum uric acid levels were positively associated with d-ROMs levels in people who underwent general health screening [22]. It was reported that d-ROMs is higher in hemodialysis patients than in normal control subjects, and d-ROMs is suggested to be a potent marker of oxidative injury that is indicative of the inflammatory status in hemodialysis patients because a positive correlation was observed between d-ROMs and CRP levels but not between MDA and CRP levels [23]. From all these studies taken together, d-ROMs seems to be a plausible marker for detecting oxidative stress in patients; however, further studies are needed to assess the implications of d-ROMs used to detect iron-induced oxidative stress in the clinical setting.

In the second part of our study, we loaded iron to mice to analyze changes in iron-induced oxidative stress in different iron storage status. Most of the iron in the body is in Hb of RBCs and the adult mouse is reported to have about 0.6–1 mg of iron in Hb [24], and iron overload inhibits erythroid burst-forming unit colony formation and erythroblast differentiation of murine and human hematopoietic progenitors in vitro [25]. To investigate changes in oxidative stress with enhancement of iron utilization through erythropoiesis, we adopted a dose of total iron loading of 0.5 mg/head iron within the dose which does not affect erythropoiesis. Serum hepcidin levels and hepatic iron content were higher in response to iron loading in the Fe(+) group than in the Fe(−) group on Day 0 (Fig. 3a, c). In contrast to the up-regulation of hepcidin by iron signaling, no change was observed in serum iron levels (Fig. 3b). Although we observed a dose-dependent increase in serum iron levels with 5 and 50 mg total iron loading in the first part of our study (Table 1), no significant change in serum iron level was observed with 0.5 mg total iron loading in the second part of our study (Fig. 3b). These results suggest that serum iron is able to be maintained at a constant level in the case of low levels of iron loading. Surprisingly, the d-ROMs level was significantly higher in the Fe(+) group than in the Fe(−) group (Fig. 3d), suggesting that d-ROMs is a sensitive marker that is able to detect oxidative stress derived even these low levels of iron loading.

To the best of our knowledge, there is no reported evidence regarding the effects of C.E.R.A. on oxidative stress through enhancement of iron utilization. The findings of our current study, showing that the oxidative stress marker d-ROMs decreased in the Fe(+) group after C.E.R.A. administration together with enhancement of iron utilization through erythropoiesis, suggest that C.E.R.A. has the potential to mobilize substantial amounts of iron from iron storage, which also accounts for the observed tendency for d-ROMs levels to decrease after C.E.R.A. treatment in the Fe(−) group (Fig. 6). In fact, the decrease in hepatic iron content in response to C.E.R.A. treatment was much greater in the Fe(+) group than in the Fe(−) group, and serum hepcidin level was higher in the Fe(+) group than in the Fe(−) group (Fig. 5; Table 3). Hepcidin inhibits iron efflux by directly binding to the iron exporter ferroportin [26]; therefore, these results imply that absorption of dietary iron in the duodenum may be suppressed in the iron-loaded group and that the usage of dietary iron for erythropoiesis would be much lower in the Fe(+) group than in the Fe(−) group. Therefore, the C.E.R.A.-treated Fe(+) group would have mobilized more iron from iron storage; consequently, a significant reduction of d-ROMs might be observed only in iron-loaded mice.

The populations of $TER119^{positive}/CD71^{high}$ cells in bone marrow cells, which mainly represent basophilic erythroblasts that are reported to incorporate iron for Hb synthesis [27, 28], were higher in C.E.R.A.-treated mice on Day 8, indicating that the demand of iron for erythropoiesis was increased under this condition (Fig. 4b). Reticulocyte counts, RBC counts, and Hb levels were also all higher in C.E.R.A.-treated mice on Day 8 (Fig. 4c; Table 3), suggesting that erythropoiesis was activated after C.E.R.A. treatment.

Hepcidin levels were lower in C.E.R.A.-treated mice on Day 1 and on Day 8 (Tables 2, 3). It has been reported that systemic hepcidin level is down-regulated by ESA treatment mainly in an indirect manner, especially via erythropoietic activity [13, 29]. Erythroferrone has also recently been reported to suppress hepcidin levels in response to erythropoiesis [30, 31]. Several groups have reported that hepcidin levels are decreased after C.E.R.A. treatment [32, 33]; however, the underlying mechanisms have not been elucidated. As shown in Table 2, *erythroferrone* mRNA expression levels were higher and hepcidin levels were significantly lower after C.E.R.A. treatment in both the Fe(−) and Fe(+) groups on Day 1, suggesting that hepcidin might be down-regulated in response to enhanced erythropoietic activity after C.E.R.A. treatment. However, the mobilization of iron from hepatic iron storage to the erythropoietic progenitor cells has not yet been clearly observed at this moment (Table 2).

In our study, C.E.R.A. markedly decreased serum iron levels on Day 8, which reflects a condition of transient iron insufficiency for erythropoiesis, a result consistent with that from the population analysis of $TER119^{positive}/$

$CD71^{high}$ cells in bone marrow cells which implies the increase of iron demand during erythroblast differentiation (Table 3; Fig. 4b). As shown in Figs. 4 and 5 and Table 3, reticulocyte counts, RBC counts, and Hb levels increased following C.E.R.A. treatment, whereas on the other hand, hepatic iron content and hemosiderin deposition decreased. Regarding the maturation process of erythrocytes, these data suggest that iron might be mobilized from iron storage to Hb in response to erythropoiesis after C.E.R.A. treatment and, consequently, the reduction of iron in iron storage contributed to the amelioration of oxidative stress. We also demonstrated that an iron-deficient diet leading to the reduction of hepatic iron storage ameliorated oxidative stress, supporting the evidence that change in d-ROMs levels occurs via iron mobilization (Fig. 7). Under these conditions, there was no difference between the iron-deficient diet group and the control diet group in terms of reticulocyte count or RBC count, suggesting that, in contrast to C.E.R.A. treatment, iron was not distributed to RBCs by this iron intervention.

Excess iron accumulation causes organ dysfunction by the production of ROS through the Fenton response and the Haber–Weiss response [34, 35]. Iron overload has been associated with carcinogenesis both in human and animal studies [17]. Several iron overload diseases, including hereditary hemochromatosis and thalassemia, demonstrate ectopic iron accumulation with consequent complications of iron-related organ damage such as cardiomyopathy, liver cirrhosis, and diabetes mellitus caused by disturbance of pancreatic insulin secretion [36]. It has been suggested that reducing increased body iron would be the most helpful strategy for preventing these disease. The results of our present study indicate that there is a possibility that measuring d-ROMs may help in the early detection of iron-induced tissue injuries and identify the status of disease progression in such individuals, and may be a useful marker for preventing iron overload disorders. Further careful investigations are needed to evaluate d-ROMs for the early detection of iron overload disease-related complications in clinical application.

Increased mortality in hemodialysis patients is largely linked to high levels of stored iron as indicated by high serum ferritin levels [37, 38]. One study reports that serum hepcidin levels were higher in maintenance hemodialysis patients than in healthy volunteers, and suggests that many of these patients were unable to effectively utilize iron storage for erythropoiesis [39]. High levels of serum hepcidin are related to increased incidence of cardiovascular events in chronic hemodialysis patients [40]. We previously reported that, compared to recombinant human erythropoietin (rhEPO), C.E.R.A. potentially has a more sustained effect on the reduction of serum hepcidin levels [13], which suggests that C.E.R.A. more efficiently promotes utilization of iron for erythropoiesis and results in a decrease in the amount of stored iron with suppressed oxidative stress. C.E.R.A. may have beneficial implications for improving prognosis by correcting iron metabolism disorders together with reducing oxidative stress.

In summary, we demonstrated that d-ROMs was a sensitive marker of iron-induced oxidative stress, and that C.E.R.A. has the potential to promote iron utilization for erythropoiesis through mobilization of stored iron to erythrocytes, consequently, resulting in a decrease in the oxidative stress marker d-ROMs in mice. Our results suggest the possibility that enhancement of iron utilization by C.E.R.A., leading to decreased oxidative stress, contributes to tissue protective properties. C.E.R.A. may have beneficial effects in improving prognosis by correcting oxidative stress-related disorders. Further investigation is needed to clarify the relationship between tissue protective effects and the amelioration of oxidative stress by enhancement of iron utilization after C.E.R.A. treatment, and it is also necessary to investigate it in clinical settings.

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Compliance with ethical standards

Conflict of interest All authors are employees of Chugai Pharmaceutical Co., Ltd.

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