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Spontaneous reduction of iron(III)porphyrin to iron(II)porphyrin–CO complex in mouse circulation

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Experimental Section

Preparation of met-hemoCD and oxy-hemoCD solutions. 5,10,15,20-Tetrakis(4-sulfonatopheyl)porphinatoiron(III) (Fe^{III}TPPS) and Py3CD were synthesized.[S1] Stock solutions of met-hemoCD used for animal experiments were prepared as follows. Fe^{III}TPPS (6.48 mg, 6.0 μmol) and Py3CD (21.12 mg, 7.2 μmol) were dissolved in PBS (1 mL) to yield a solution of met-hemoCD1 (6 mM). The solution of met-hemoCD (1.5 mM) used in the animal experiments was prepared by diluting the stock solution of met-hemoCD (6 mM) fourfold with PBS. To prepare oxy-hemoCD, the stock solution of met-hemoCD was reduced with $Na_2S_2O_4$. Excess $Na_2S_2O_4$ was removed by passing the solution through a HiTrap desalting column (Sephadex G25, GE Healthcare Life Sciences). During the column treatment, the complex became its O_2 adduct by capturing atmospheric O_2. The concentration of met-hemoCD and oxy-hemoCD was determined from its absorption coefficient $\varepsilon_{422}^{met} = 1.19 \times 10^5\ M^{-1}cm^{-1}$, $\varepsilon_{422}^{oxy} = 1.64 \times 10^5\ M^{-1}\ cm^{-1}$.[S2]

Animal experiments. The animal experiments conducted in the present study were approved by the Guidelines for Animal Experiments of Doshisha University (approved No. A22002 and A21064). The guideline “Doshisha University Regulations on the Conduct of Animal Experiments and Related Activities” based on the provisions in the Act on Welfare and Management of Animals (Act No. 105 of October 1, 1973) (hereinafter “Animal Welfare Act”), Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notice No. 88 of the Ministry of the Environment, April 28, 2006) (hereinafter “Care and Management Standards”), Standards relating to the Methods of Destruction of Animals (Notice No. 40 of the Prime Minister’s Office, July 4, 1995) (hereinafter “Animal Destruction Standards”), and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions (Notice No. 71 of the Ministry of Education, Culture, Sports, Science and Technology, June 1, 2006) (hereinafter “Animal Experiment Guidelines”), set out necessary matters for the appropriate conduct of animal experiments and related activities from the standpoints of scientific rationale and animal welfare, as well as of environmental preservation and assurance of security of faculty, staff and students engaging in animal experiments and related activities. The animal experiments were conducted according to the provisions of these regulations as well as the provisions of Animal Welfare Act, Care and Management Standards, Animal Destruction Standards, Animal Experiment Guidelines and other laws and ordinances.

(1) Methods must be employed which cause as little pain and stress as possible to the laboratory animals, while still allowing attainment of the scientific objective.

(2) Efforts must be made to replace laboratory animals with other research materials where

possible, while still allowing attainment of the scientific objective.

(3) Efforts must be made to reduce the number of animals used to as few as possible, while still allowing attainment of the scientific objective.

For further details on the guideline, refer to:
https://www.doshisha.ac.jp/research/ethics/regulations.html#animal

We housed three mice per cage under controlled environmental conditions and fed commercial feed pellets. All mice had free access to food and water. The solution of oxy-hemoCD (1.5 mM, 0.15 mL) or met-hemoCD (1.5 mM, 0.15 mL) was intraperitoneally injected to female BALB/cCrSlc mice (4–6 weeks). After injection, the mice were sacrificed by cervical dislocation at different time points. The naturally excreted urine was collected. The liver samples were excised by disposable biopsy punch (5 mm), washed with saline, and soaked in RNA later stabilization reagent (QIAGEN). These samples were stored at 4°C until use. For blood sampling, after intraperitoneally injection of the solution of met-hemoCD or oxy-hemoCD, mice were anaesthetized by isoflurane. Blood samples were collected from right ventricle and mixed with heparin. The blood samples were then stored at 4°C and used on the day of the experiment.

Urinary analysis. The calculation to determine the amount of met-, oxy-, and CO complexes of hemoCD in the collected urine samples were performed as follows.[S2] The urine samples were appropriately diluted by PBS (spectrum 1 in Figure 1) for UV-vis measurements (Shimadzu UV-2450). Then oxy-hemoCD was converted to CO-hemoCD by bubbling pure CO gas into the solution (spectrum 2 in Figure 1). Subsequently, excess $Na_2S_2O_4$ (1–2 mg) was added to the solution, all hemoCD species being converted to CO-hemoCD (spectrum 3 in Figure 1). As the UV-vis spectrum of hemoCD in urine was the same with that in PBS, the same molar extinction coefficients for met-, oxy- and CO-hemoCD were used in the urinary analysis. The concentration of total hemoCD (C_{total}) was determined from the absorbance at 422 nm of spectrum 3 by eq 1:

$$A_{422}^{3} = \varepsilon_{422}^{CO} \cdot C_{total} \cdot l \quad (1)$$

where ε_{422}^{CO} is the molar extinction coefficient at 422 nm of CO-hemoCD ($\varepsilon_{422}^{CO} = 3.71 \times 10^5\ M^{-1}cm^{-1}$). The l is the optical path length (1.0 cm). The hemoCD solution in the urine samples of spectrum 1 contains CO-hemoCD1 (C_{co}), oxy-hemoCD and met-hemoCD (C_{oxy}) (eq 2):

$$C_{total} = C_{CO} + C_{oxy} + C_{met} \quad (2)$$

The absorbance at 422 nm of spectrum 2 is defined as eq 3:

$$A_{422}{}^{2} = \varepsilon_{422}{}^{CO} \cdot (C_{total} - C_{met}) + \varepsilon_{422}{}^{met} \cdot C_{met} \quad (3)$$

where $\varepsilon_{422}{}^{met}$ is the molar extinction coefficient at 422 nm of met-hemoCD ($\varepsilon_{422}{}^{met}$ = 1.19 × 10^5 $M^{-1}cm^{-1}$). Therefore, the concentration of met-hemoCD (C_{met}) is represented as eq 4:

$$C_{met} = \frac{A_{422}{}^{3} - A_{422}{}^{2}}{\varepsilon_{422}{}^{CO} - \varepsilon_{422}{}^{met}} \quad (4)$$

The absorbance at 422 nm of spectrum 1 is described as eq 5:

$$A_{422}{}^{1} = \varepsilon_{422}{}^{CO} \cdot C_{CO} + \varepsilon_{422}{}^{oxy} \cdot C_{oxy} + \varepsilon_{422}{}^{met} \cdot C_{met} \quad (5)$$

Therefore, the concentration of oxy-hemoCD (C_{oxy}) is represented as eq 6:

$$C_{oxy} = \frac{A_{422}{}^{2} - A_{422}{}^{1}}{\varepsilon_{422}{}^{CO} - \varepsilon_{422}{}^{oxy}} \quad (6)$$

where $\varepsilon_{422}{}^{oxy}$ is the molar extinction coefficient at 422 nm of oxy-hemoCD ($\varepsilon_{422}{}^{oxy}$ = 1.64 × 10^5 $M^{-1}cm^{-1}$). The concentration of CO-hemoCD (C_{CO}) is represented as eq 7:

$$C_{CO} = \frac{A_{422}{}^{3}}{\varepsilon_{422}{}^{CO}} - C_{oxy} - C_{met} \quad (7)$$

The moles of met-, oxy-, and CO complexes in total hemoCD (M_X, X = met, oxy or CO M_{oxy} and M_{CO}) are determined as eq 8:

$$M_X = C_X \cdot V \quad (8)$$

where V is the volume of the collected urine samples.

Measurement of CO-Hb%. The isolation of RBC was performed according to the literature.[S3] Blood samples was centrifuged at 2000 rpm for 5 min to remove plasma, the remaining RBC components were diluted in PBS (1:2, v/v). The solution was centrifuged at 2000 rpm for 5 min, and then washed with PBS for 2 times to isolate the RBC. CO-Hb% in whole blood or RBC was quantified according to the reported method.[S4] Briefly, whole blood (15 μL) and

RBC (10 μL) from healthy BALB/cCrSlc mice were diluted with 0.1% Na_2CO_3 solution and pure O_2 gas was bubbled into this solution for 5 min. Then, excess $Na_2S_2O_4$ was added to the solution and the absorbance at A_{540} and A_{579} were measured by UV-vis spectroscopy (Shimadzu UV-2450). The A_{540}/A_{579} values were calculated as A_0. RBC (7.5 μL) or whole blood (7.5 μL) from healthy BALB/cCrSlc mice were diluted with 0.1% Na_2CO_3 solution, and bubbled by pure CO gas for 1 min. Excess $Na_2S_2O_4$ was added to the solution and the absorbance at A_{540} and A_{579} were measured. The A_{540}/A_{579} values were calculated as A_{100}. RBC (7.5 μL) or whole blood (7.5 μL) collected from oxy- or met-hemoCD-treated mice were diluted with 0.1% Na_2CO_3 solution and excess $Na_2S_2O_4$ was added. The A_{540}/A_{579} values were calculated as A_X. The CO-Hb% values were obtained using eq 9:

$$\text{CO-Hb \%} = \frac{A_X - A_0}{A_{100} - A_0} \times 100 \tag{9}$$

RT-PCR. Total RNA was isolated from the mouse livers using the RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions. The RNA concentration and purity were determined by measuring the absorbances at 260 and 280 nm (Shimadzu BioSpec-nano). The resulting total RNA (500 ng) was reverse transcribed in a mixture (8 μL) of 5 × RT Master Mix (Toyobo, 2 μL), RNase free water. The sample was incubated at 37°C for 15 min, at 50°C for 5 min, and at 98°C for 5 min. Real-time PCR was performed in 9 μL volumes using MicroAmp™ Fast Optical 48-well reaction plates (Applied Biosystems) for each well set on a Step One real-time PCR system (Applied Biosystems). The Taqman™ Gene Expression Assays (Applied Biosystems) used are as follows: β-actin (assay ID: Mm00607939_s1), Hmox-1 (assay ID: Mm00516005_m1), ALAS-1 (assay ID: Mm01235914_m1). The PCR mixture was as follows: cDNA (2 μL), TaqMan™ Fast Advanced Master Mix (10 μL), Taqman™ Gene Expression Assays (2 μL), Nuclease-free Water (7μL). β-actin was used as a reference gene. Forty cycles (95°C for 20 s, 95°C for 1 s, 60°C for 20 s, 95°C for 1 s, 60°C for 20 s) of PCR were performed after 10 min of denaturation at 95°C. All samples were measured in triplicate. Data were analyzed by the comparative C_T method ($\Delta\Delta C_t$) using StepOne software v2.3 (Applied Biosystems).

Statistical analysis. All data represent the means ± standard error from at least three different experiments and were analyzed by Student's t-test. Differences with *P* values of less than 0.05 were considered significant.

References

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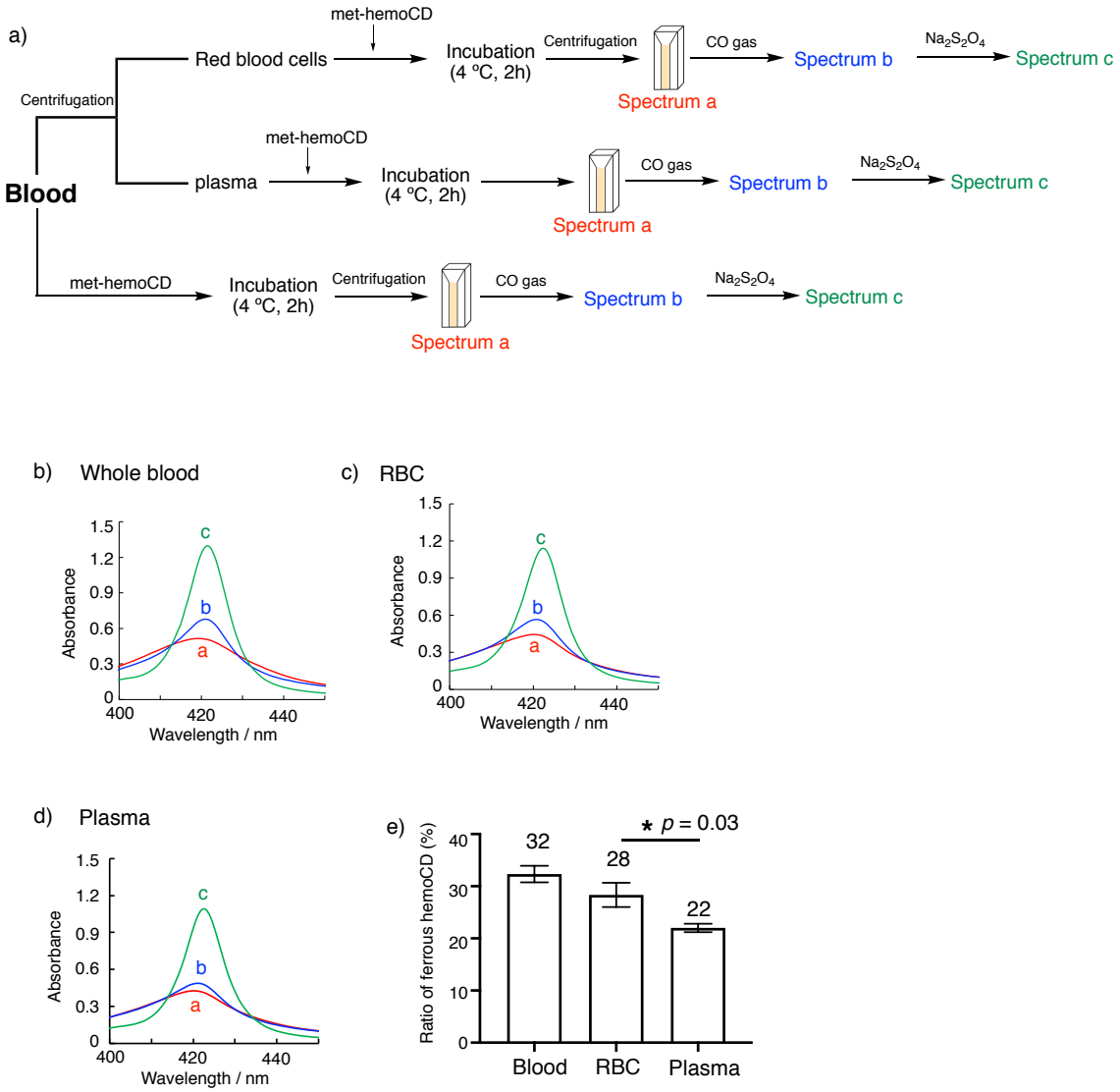

Fig. S1. Reduction of the iron(III) center of met-hemoCD by mixing with whole blood, RBCs or plasma *ex vivo.* (a) Whole blood (100 μL), RBCs (100 μL) and plasma (100 μL) collected from healthy BALB/cCrSlc mice were mixed with met-hemoCD (0.4 mM, 50 μL) and incubated at 4°C. After 2 h, the hemoCD component was separated by centrifugation and the supernatant was appropriately diluted by PBS for UV-vis measurements. The determination on the amount of met-, oxy-, and CO complexes of hemoCD in the solution were performed as the same method for the urinary analysis. (b-d) UV-vis spectra of diluted hemoCD in PBS (spectrum a) and after addition of CO gas (spectrum b) and $Na_2S_2O_4$ (spectrum c). (e) The ratio of ferrous hemoCD in the collected hemoCD components. Each bar represents the means ± standard error (n = 3–6 mice per group).