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Red Blood Cells Donate Electrons to Methylene Blue Mediated Chemical Reduction of Methemoglobin Compartmentalized in Liposomes in Blood

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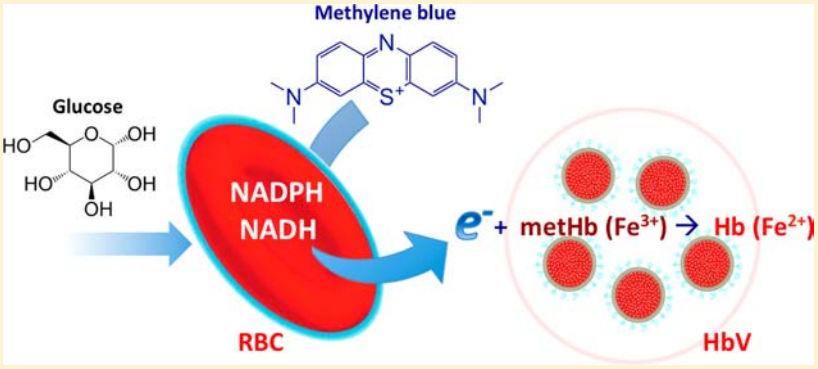

ABSTRACT: Electron-energy-rich coenzymes in cells, NADH and NADPH, are re-energized repeatedly through the Embden−Meyerhof and pentose−phosphate glycolytic pathways, respectively. This study demonstrates extraction of their electron energies in red blood cells (RBCs) for in vivo extracellular chemical reactions using an electron mediator shuttling across the biomembrane. Hemoglobin-vesicles (HbVs) are an artificial oxygen carrier encapsulating purified and concentrated Hb solution in liposomes. Because of the absence of a metHb-reducing enzymatic system in HbV, HbO_2 gradually autoxidizes to form metHb. Wistar rats received HbV suspension (10 mL/kg body weight) intravenously. At the metHb level of around 50%, methylene blue [MB^+; 3,7-bis(dimethylamino)phenothiazinium chloride] was injected. The level of metHb quickly decreased to around 16% in 40 min, remaining for more than 5 h. In vitro mixing of HbV/MB^+ with RBCs recreated the in vivo metHb reduction, but not with plasma. NAD(P)H levels in RBCs decreased after metHb reduction. The addition of glucose facilitated metHb reduction. Liposome-encapsulated NAD(P)H, a model of RBC, reduced metHb in HbV in the presence of MB^+. These results indicate that (i) NAD(P)H in RBCs reacts with MB^+ to convert it to leukomethylene blue (MBH); (ii) MB^+ and MBH shuttle freely between RBC and HbV across the hydrophobic lipid membranes; and (iii) MBH is transferred into HbV and reduces metHb in HbV. Four other electron mediators with appropriate redox potentials appeared to be as effective as MB^+ was, indicating the possibility for further optimization of electron mediators. We established an indirect enzymatic metHb reducing system for HbV using unlimited endogenous electrons created in RBCs in combination with an effective electron mediator that prolongs the functional lifespan of HbV in blood circulation.

INTRODUCTION

NADH, NADPH, and their oxidized forms, NAD^+ and $NADP^+$, are electron mediators of various biological processes including energy metabolism, mitochondrial functions, calcium homeostasis, antioxidation/generation of oxidative stress, gene expression, immunological functions, aging, and cell death.[1,2] Within circulating red blood cells (RBCs) in the bloodstream, these mediators are key elements for glycolysis and antioxidative functions.[3] During a long history of evolution, mammalian RBCs have lost the nuclei to focus their functions to oxygen transport and glycolysis, which are important to maintain the metabolism of the host body and protect it from oxidative damage. During the glycolysis, high electron energies are produced and charged repeatedly as NADPH and NADH. $NADP^+$ is reduced to NADPH by glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) in the pentose−phosphate pathway. NADPH is used mainly for glutathione reductase and NADPH-flavin metHb reductase,[4] both for protection from oxidative damage. NAD^+ is reduced to NADH by glyceraldehyde 3-phosphate

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dehydrogenase (GAPDH) in the Embden−Meyerhof pathway. NADH is used mainly for NADH-Cytochrome b_5 reductase to reduce metHb,[5] and for lactate dehydrogenase to convert pyruvate to lactate.[6] Consequently, the electron energies of NADH and NADPH are consumed within RBCs. We have developed the idea of extracting these intracellular electron energies across the biomembrane and of using them for extracellular chemical reactions in blood circulation. This is a method of reduction of metHb in artificial oxygen carriers.

Hemoglobin-based oxygen carriers (HBOCs) have been studied worldwide for clinical use because they are expected to alleviate transfusion-related problems such as (i) the possibility of infection (HIV, hepatitis, etc.) because of a "window period" in which the amount of virus is too low to detect, (ii) the possibility of blood-type mismatching by human error, and (iii) the possibility of blood shortages in an emergency situation because of the short shelf life of donated blood (3−6 weeks refrigerated).[7−10] Hemoglobin-vesicle (HbV) is an HBOC encapsulating purified human Hb solution in a phospholipid vesicle (liposome) to shield toxic effects of cell-free Hbs.[10−12] Earlier studies clarified the safety and efficacy of HbVs as a transfusion alternative and for oxygen therapeutics using animal models.[13−20] Actually, HbVs do not contain the metHb-reducing enzymatic system that is present in the original RBCs. Hb is purified from outdated human blood by pasteurization (60 °C, 12 h) to achieve the utmost safety from any possibility of infection.[21] During this procedure, all the unstable enzymes, including the metHb-reducing system, are denatured and eliminated. As a result, Hb in HbV gradually increases the metHb level after intravenous injection. MetHb in HbV cannot be reduced by plasma ascorbic acid (AsA) because of the high barrier functionality of the lipid bilayer membrane of HbV with no ionophore. We attempted to create some nonenzymatic systems within HbV to reduce metHb.[22−27] However, these methods are not practical because of the systemic complexity and limited efficacy for metHb reduction.

In fact, our pilot in vivo study (see Figure 1 in this paper) of intravenous single injection of methylene blue [MB^+; 3,7-bis(dimethylamino)phenothiazinium chloride] following HbV injection demonstrated the effective reduction of metHb in HbV. MB^+ is a water-soluble phenothiazine dye that is commonly used as one component of the Giemsa stain solution in pathological studies, an electron mediator in electrochemical reactions, and therapeutic drugs for neurological disease, methemoglobinemia, and so forth.[28−30] Because HbV contains no potential electron donor, this phenomenon implies that the injected MB^+ in plasma was somehow reduced by biological electron donors, possibly those in RBCs. The reduced leukomethylene blue (MBH) penetrated across the lipid bilayer membrane of HbV to reduce metHb. This study was undertaken to identify the source of electrons to reduce metHb in HbV and to clarify all electron pathways. We also studied its long-term effectiveness for in vivo studies using MB^+ and the possibility of other potential electron mediators.

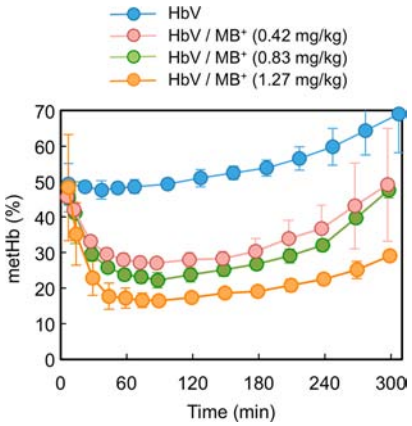

Figure 1. Time course of the level of metHb in HbV after intravenous administration into Wistar rats (n = 3, mean ± SD). HbV with the metHb level of 50% was injected. Without MB^+, the level increased gradually. Subsequent MB^+ injection showed dramatic reduction of the level of metHb, depending on the amount of MB^+.

RESULTS

In Vivo Reduction of metHb in HbV by Injection of MB^+. HbV with the metHb level of nearly 50% was injected into the rat femoral vein at a dose rate of 10 mL/kg, which corresponded to about 18% of whole blood volume (56 mL/kg). Subsequent injection of MB^+ (0.42 mg/kg) rapidly decreased the level of metHb to about 30%, but it returned to a gradual increase (Figure 1). With increasing the amount of MB^+ to 0.83 and 1.27 mg/kg, the level of metHb decreased. It was 16% at 60 min after injection of 1.27 mg/kg MB^+. Accordingly, the reduction of metHb is dependent on the dose amount of MB^+. The low level of metHb was sustained for over 6 h by injecting 1.27 mg MB^+/kg body weight.

In Vitro Reduction of metHb in HbV by MB^+ and RBCs. Both fresh rat whole blood and washed RBCs in the presence of MB^+ showed an effective metHb reduction in HbV from 50% to 10−15% in 5 min, and the low metHb level was sustained for 60 min (Figure 2). With decreasing amount of washed RBCs, the metHb reduction is minimized. Rat plasma alone showed no metHb reduction at all. These results indicate that electron donors are present in RBCs, but not in plasma,

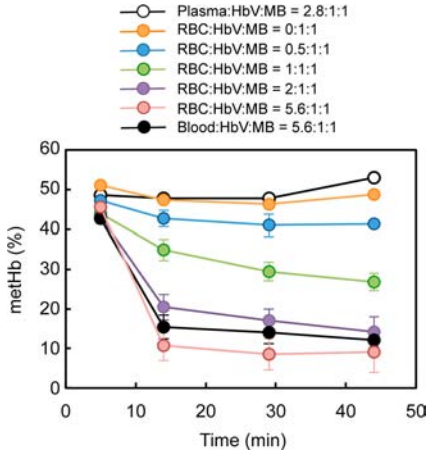

Figure 2. In vitro reduction of metHb in HbV by the addition of blood components and MB^+ (n = 3, mean ± SD). "Blood" was fresh whole blood collected from Wistar rats and heparinized. "Plasma" was obtained by centrifugation of whole blood. The whole blood [Hb] was around 12 g/dL. "RBC" was washed RBCs. The mixing ratio, blood:HbV:MB = 5.6:1:1 by vol corresponds to the in vivo experiment of injection of 10 mL/kg HbV and 10 mL/kg MB^+ to a Wistar rat that possesses blood volume of 56 mL/kg. Because the hematocrit of whole blood was around 50%, the mixing ratio of plasma:HbV:MB was adjusted to 2.8:1:1 by vol. The presence of RBCs contributes to the metHb reduction in HbV.

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and that the MB^+-MBH redox system works as a mediator to transport electrons to metHb in HbVs. Both levels of the reduced forms, NADH and NADPH, significantly decreased in RBCs after metHb reduction in HbV (Figure 3), indicating that NADH and NADPH would be the electron donors.

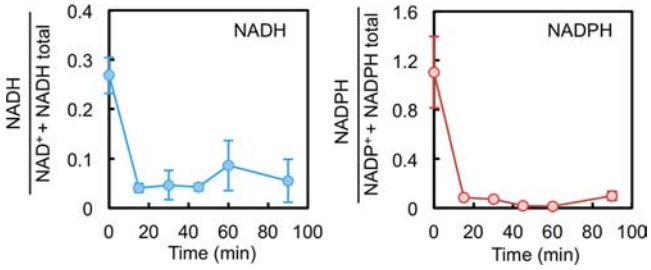

Figure 3. Decreased levels of NADH and NADPH in rat RBCs after the reduction of metHb in HbV in the presence of MB^+ (n = 3, mean ± SE).

External addition of α-D-glucose to washed RBCs at the beginning showed effective metHb reduction of HbV for over 4 h (Figure 4). However, without α-D-glucose, the level of metHb

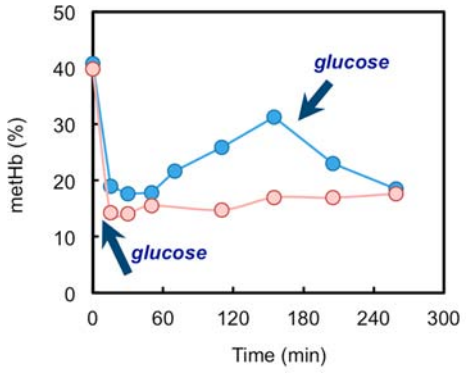

Figure 4. Addition of α-D-glucose to washed RBCs affects the metHb reduction in HbV in the presence of MB^+. The mixing ratio RBC:HbV:MB:glucose = 2:1:1:1 by vol. The pink plots show that the addition of α-D-glucose from the beginning exhibits more effective and durable metHb reduction in HbV. Blue plots show that a late addition of α-D-glucose improves metHb reduction in HbV.

increased gradually. Addition of α-D-glucose after 3 h dramatically facilitated the metHb reduction. These results demonstrate that the concentration of electron donors in RBCs was increased by the facilitated glycolysis in RBCs.

Partition Experiment of MB^+ in HbV and RBC. This experiment was performed to determine the percentage of MB^+ binding to the surface or into the membrane of the HbV or rat RBC. The results of MB^+ partition into HbV showed no obvious dependence on the mixing volume ratio of MB^+/HbV (0.1−2.0), and the percentage of MB^+ incorporated into HbV was 37.7 ± 2.9% (mean ± SD). The same tendency was confirmed with rat RBCs, and the percentage of MB^+ incorporated into rat RBCs was 35.5 ± 6.3%.

In Vitro Reduction of metHb in HbV by MB^+ and Chemical Reagents. MB^+ has characteristic absorption with the λ_{max} at 650 nm. It decreases with the addition of an electron donor (Figure 5). Addition of NADPH and NADH showed rapid reduction of MB^+ to MBH. The reaction completed within 20 min. Cys completed the reaction within 40 min. GSH

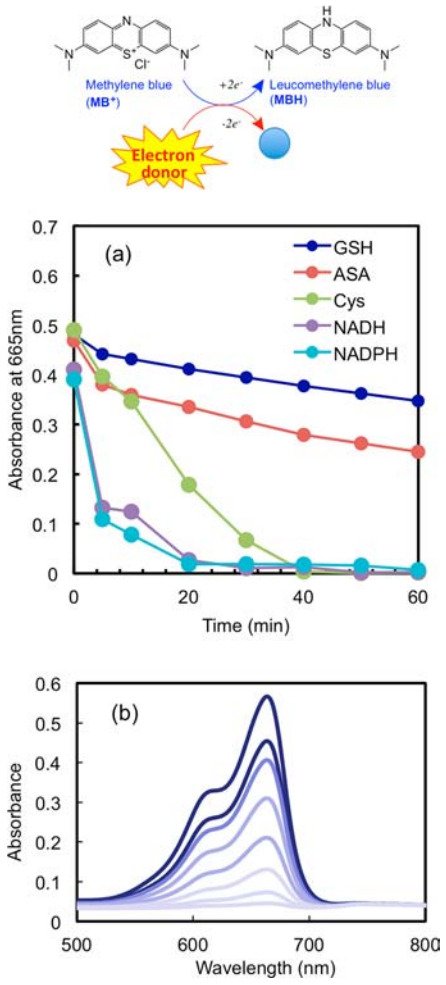

Figure 5. (a) Time course of MB^+ reduction by a reductant (AsA, GSH, Cys, NADH, or NADPH) in anaerobic conditions in pH 7.4 phosphate buffered saline. The reaction mixture in a total of 5 mL contained 6.8 μM of MB and 1000 μM of one electron donor. (b) Change of absorption spectra of MB^+ during reduction by Cys (0 → 60 min).

and ASA showed extremely slow reduction. The reduction was not completed even after 60 min.

Liposome Encapsulated NADPH or NADH as a Model of RBCs Can Reduce metHb in HbV. All the liposome encapsulated NADPH or NADH showed rapid reduction of metHb in HbV by the addition of MB^+ (Figure 6), which indicates that MB^+ permeates across the lipid membrane into the NADPH-liposome or NADH-liposome and that MB^+ is reduced to form MBH, which diffuses out from the NADPH-liposome or NADH-liposome and then enters into HbV to reduce metHb in HbV. The phase transition temperature of DPPC is 41 °C; that of DMPC is 23 °C. Therefore, the lipid bilayer membrane containing DPPC is expected to possess a generally lower molecular fluidity and a higher barrier functionality than those of DMPC. However, no marked difference was observed between DPPC and DMPC liposomes for the reduction of metHb in HbV, indicating that MB^+ and MBH freely shuttle across the lipid bilayer membranes of both liposomes.

Practical in Vivo Study of HbV and MB^+. Injection of HbV alone (metHb less than 5%) into Wistar rats showed a gradual metHb increase, as shown in Figure 7. Co-injection of

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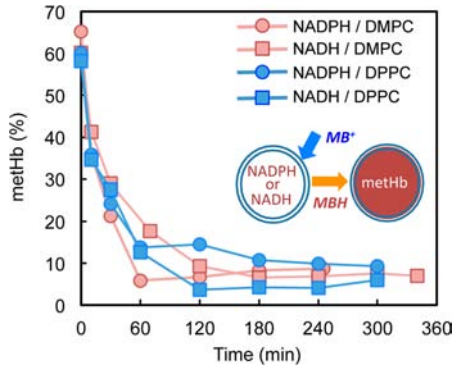

Figure 6. Reduction of metHb in HbV by the addition of MB^+ and liposome-encapsulated NADPH or NADH in an anaerobic condition. The liposome membranes consist of DPPC/cholesterol/DHSG/PEG_{5000}-DSPE or DMPC/cholesterol/DHSG/PEG_{5000}-DSPE = 5/4/0.9/0.03 by mol. All the liposomes showed reduction of metHb in HbV by the addition of MB^+.

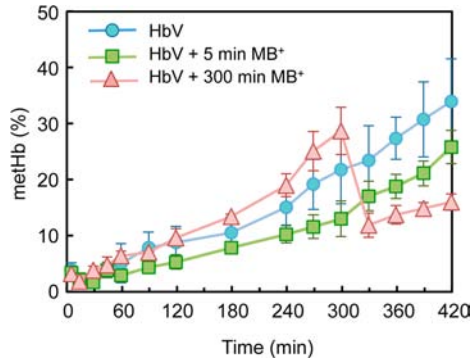

Figure 7. Time course of the level of metHb in HbV after intravenous administration into Wistar rats (n = 3, mean ± SD). HbV with the metHb level of <5% was injected. Without MB^+, the level increased gradually. Co-injection of MB^+ showed a lower rate of metHb increase. Late MB^+ injection at around 300 min showed significant metHb reduction.

MB^+ at the beginning showed a slowed increase in metHb. Injection of MB^+ at 5 h showed the rapid reduction of metHb. Accordingly, MB^+ injection can improve the functional life span of HbV.

Other Electron Mediators for metHb Reduction in HbV. Among the other electron mediators tested, AZA, BCB, TLB, and TDB showed similar metHb reduction to that of MB^+ (Figure 8). However, PM tended to return to a higher metHb level and TA showed insufficient metHb reduction.

■ DISCUSSION

Our primary finding is that the high electron energies present within RBCs are used effectively for extracellular chemical reaction, namely, reduction of metHb compartmentalized in HbV in circulating blood of Wistar rats. Here we propose the main electron pathway. The injected MB^+ in plasma diffuses across the biomembrane of RBCs, and NADPH and NADH in RBCs reduce MB^+ to MBH, which permeates across the hydrophobic lipid bilayer membranes of both RBC and HbV, and transfers an electron to metHb in HbV. Thus, NADPH and NADH provide electrons to metHb in HbV via the shuttling MB^+-MBH redox system (Scheme 1).

We have studied physicochemical and biochemical characterization,[11,12,43] safety,[15−17] and efficacy[13,14,18,19] of HbV for

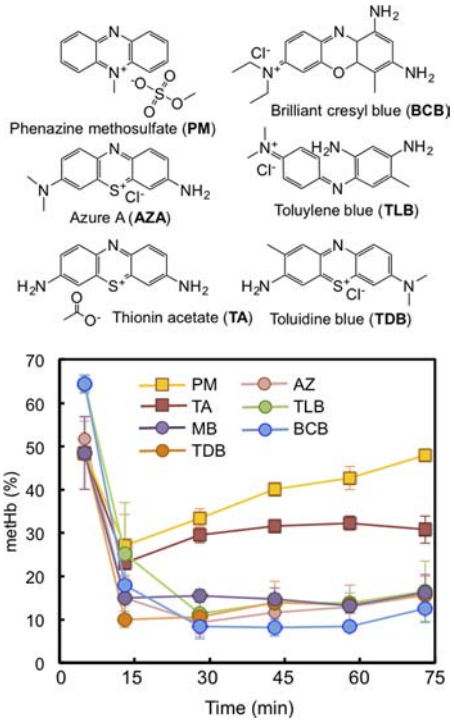

Figure 8. In vitro reduction of metHb in HbV by the addition of RBCs and a series of potential electron mediators. The mixing volume ratio of RBC:HbV:electron mediator = 5.6:1:1. The concentration of an electron mediator solution was adjusted to 0.35 mM. [Hb] of the RBC suspension was adjusted at 12 g/dL. HbV suspension contained about 50% metHb. Among six potential electron mediators, AZA, BCB, TLB, and TDB showed effective metHb reduction as did MB^+. However, PM and TA showed less effectiveness (n = 3, mean ± SD).

clinical usage as a transfusion alternative and oxygen therapeutic. HbVs contain no metHb reducing enzymatic system that exists in original RBCs. Hb is purified from outdated human blood by pasteurization to ensure the utmost safety from infection.[21] This strategy contrasts against others by maintaining the original metHb reducing system in red blood cells.[31,32] During our Hb purification procedure, all enzymes including the metHb-reducing system are denatured and eliminated. Therefore, Hb in HbV gradually increases the metHb level and loses its oxygen carrying capacity. One important point is that encapsulation of Hb in liposomes can shield all the toxic effects of the further oxidization to ferrylhemoglobin and iron release induced by the addition of excess amount of hydrogen peroxide.[33] On the other hand, to prolong the functional life span of HbV, establishment of a metHb reducing system has long been a challenging subject in our research group.[24,34] To reduce the level of metHb in HbV, we tested coencapsulation of a series of thiols[23] and catalase,[25] photoreduction using flavin,[27] construction of catalase-like activity using metHb and tyrosine,[26] and so forth. Unfortunately, these methods are not practical because of the low efficiency and the addition of other new components to encapsulate into HbVs, which complicates the whole preparation for clinical use.

In the case of cell-free chemically modified Hb solutions such as intramolecular cross-linked Hb and polymerized Hb, the rate of autoxidation is reduced in the bloodstream because metHb can react directly with reducing agents that are present in plasma, such as AsA.[35−38] Nearly 100 μM of AsA is present in

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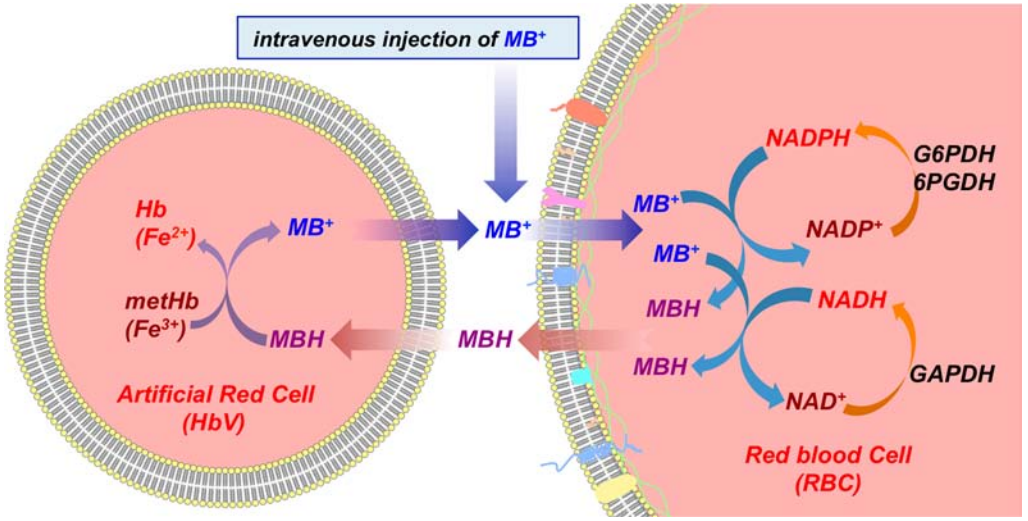
Scheme 1. Proposed Electron Transfer Pathway from RBCs to metHb in HbV[a]



[a]Intravenously injected MB^+ is first dispersed in plasma of blood. MB^+ permeates across the biomembrane of RBCs to the cytoplasmic space, where it reacts with NADPH and NADH to be reduced to MBH. The oxidized $NADP^+$ is energized repeatedly to NADPH using 6-phosphogluconate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) in the pentose phosphate pathway. The oxidized NAD^+ is energized repeatedly to NADH by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the Embden−Meyerhof pathway. The reduced MBH permeates across the biomembrane of RBCs to the extracellular phase and diffuses across the lipid bilayer membrane of HbV and transfers an electron to ferric metHb to form ferrous Hb. The oxidized MB^+ again diffuses out across the lipid bilayer membrane of HbV and across the biomembrane of RBCs repeatedly.

healthy rat blood plasma.[39] However, in the case of HbV, Hb is shielded by the lipid bilayer (liposome) so that AsA cannot permeate and thereby reduce metHb in HbV. In our rat pilot experiment, the injection of MB^+ alone reduced the metHb level in HbV effectively from 50% to around 16%, at doses of 10 mL/kg, which correspond to 18 vol % of circulating blood volume. To clarify the mechanism of metHb reduction, some in vitro experiments were conducted using fresh rat blood, plasma, and washed RBCs. In the rat blood and washed RBC suspension, the reduction of metHb in HbV was observed equally in the presence of MB^+. However, reduction was not observed in the rat plasma. The concentration of AsA in rat plasma, around 100 μM,[39] would be insufficient to reduce MB^+ rapidly. Our result, which showed that MB^+ is reduced directly by RBCs, clearly illustrates that the main electron donor is present in RBCs. We specify that the electron donors in RBCs are NADPH and NADH based on the following reasons: (1) The level of NADPH and NADH decreased after metHb reduction. (2) Increased glycolysis facilitated metHb reduction in HbV. (3) The reduction of MB^+ by NADPH and NADH are much faster than by AsA or GSH. (4) Liposomes encapsulated NADPH and NADH, a model of RBCs, showed effective metHb reduction. (5) Oxidation−reduction potentials (E'_0 at 20 °C, pH 7.0) of Hb (Fe^{3+}/Fe^{2+}), MB^+/MBH, and $NAD(P)^+$/NAD(P)H are +0.14, +0.01, and −0.32 V, respectively.[40] The direction of electron transfer from NAD(P)H to metHb via MB^+ is reasonable.

Results of a partition experiment revealed that nearly 35−40% of MB^+ are incorporated in both RBC and HbV lipid membranes. Ionic interaction between the positively charged MB^+ and the negatively charged lipid membranes of both HbV and RBC would facilitate the incorporation of MB^+ into the hydrophobic lipid membranes.[41] Judging from the chemical structure, the reduced form MBH is more easily incorporated into the lipid membranes. Actually, liposome-encapsulated NADPH and NADH mixed with MB^+ reduced metHb in HbV effectively. No marked difference was found between DPPC (T_c = 41 °C) and DMPC (T_c = 23 °C) liposomes, which should differ in terms of membrane fluidity.[42] This result would correspond to the fast MB^+ uptake into both liposomes. In fact, we tried to measure the rate of MB^+ incorporation into the DPPC- and DMPC-containing liposomes. Because of the technical difficulty in the ultracentrifuge, we could not track the entire incorporation profile, but it was clarified that MB^+ incorporation was quite fast and equilibrated within a few minutes. Both the NAD(P)H-encapsulated liposomes and HbV bear PEG chains on their surface, which would prevent direct contact with each other.[43] PEG-modified HbV would not directly contact RBCs bearing glycocalyx layer on the surface. Therefore, the experimental results suggest that both MB^+ and MBH freely move across the hydrophobic lipid membranes of both RBC and HbV and diffuse through aqueous phase between the membranes.

MB^+ [3,7-bis(dimethylamino)phenothiazinium chloride], a water-soluble phenothiazine dye, has been used clinically for the primary and standard therapy of methemoglobinemia.[28] MB^+ is also used for photochemical virus inactivation of biological products,[44] therapeutic drug for a rescue of destructive shock,[29] as an antimalarial agent,[45] for a treatment of a neurological disorder such as Alzheimer's disease,[46] and vasoplegia during cardiac surgery with cardiopulmonary bypass[30] as an elective inhibitor of nitric oxide cyclic guanosine monophosphate. The safety of MB^+ injection is studied extensively, especially in hemodynamic and vascular endothelial functions.[28,47,48] According to the instruction of commercially available "methylenblau VITIS i.v." (Neopharma GmbH & Ci. KG), the maximum dosage of MB^+ to human for single dosage is 2 mg/kg. The repeated injection should be less than 7 mg/kg because of various side effects.[34] In our experiment, the maximum dosage of MB^+ was 1.3 mg/kg, which was much lower than the limited dosage.

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In the case of rescue from methemoglobinemia,[28,49] it has been believed that hydrophilic MB^+ in blood after intravenous administration would be reduced to MBH by RBC plasma membrane NADPH reductase or NADPH-dependent metHb reductase, and the less hydrophilic MBH would permeate across the hydrophobic biomembrane and be incorporated in RBCs. Then MBH would nonenzymatically transfer electron to intracellular metHb in RBCs. No extracellular reaction had been considered. In contrast, our results show clearly that MB^+ reacts easily and directly with NADPH and NADH aqueous solutions and liposome-encapsulated NADPH and NADH with no enzyme. Only re-energizing $NADP^+$ and NAD^+ requires glycolytic enzymatic reactions in RBCs. So-called plasma membrane oxidoreductases[50] would not be required in our system.

MB^+ can be reduced by AsA and GSH[51,52] although the reaction rate is much lower than that of either NADPH or NADH, as shown in Figure 5a, indicating that AsA and GSH within RBCs can contribute, in part, as electron donors to the reduction of MB^+. However, GSH in RBCs is used for reduction of oxidized AsA, dehydro ascorbic acid (DHA), and the oxidized GSH (GSSG) is reduced by NADPH-dependent glutathione reductase.[3] AsA is used as a reactive oxygen scavenger. The resulting mono dehydro ascorbic acid (MDA) is reduced to AsA by NADH-dependent MDA reductase. Consequently, all electrons originate from NADPH and NADH, the oxidized forms of which are re-energized repeatedly during glycolysis.

The defense mechanism to oxidative stress is different between animal species. Biosynthesis of ascorbic acid is present in rats but not in humans and guinea pigs and the superoxide dismutase activity is higher in humans and guinea pigs than in rats.[53] The plasma ascorbic acid level is higher in rats than in humans and guinea pigs.[39] Oxidized dehydroascorbate is reduced by cytochrome *b*561, one plasma membrane oxidoreductase, residing on human and guinea pig RBCs but not on rat RBCs.[54] In fact, we conducted some in vitro experiments using blood from humans and guinea pigs, and it was clarified that both human and guinea pig RBCs reduced metHb in HbV in the presence of MB^+ as did rat RBCs (data not shown here). Therefore, we believe that all mammalian RBCs with NAD(P)H are a potent electron donor which is created by the glycolytic mechanism, and the evolutional difference in ascorbic acid biosynthesis does not affect our results.

Intravenously injected MB^+ in plasma would directly contact not only RBCs, but also endothelial cells in the vasculature, where MB^+ might be reduced by NADPH and NADH in the endothelial cells. The reduced MBH would come out to the plasma phase and would be expected to contribute to metHb reduction in HbV. However, we infer that RBCs are the main source of the electron donor to metHb in HbV because the in vitro mixing experiment using blood (Figure 2) recreates in vivo results.

We tested other potential electron mediators:[55−57] phenothiazines (AZA, E'_0 = 0.013 V; TA, E'_0 = 0.063 V; TDB, E'_0 = 0.011 V), indamine (TLB, E'_0 = 0.115 V), phenazine (PM, E'_0 = 0.080 V), and phenazonium (BCB, E'_0 = 0.047 V). Their E'_0 values are similar to that of MB^+ (+0.01 V) and are intermediate between those of $Hb(Fe^{3+}/Fe^{2+})$ (+0.14 V) and $NAD(P)^+/NAD(P)H$ (−0.32 V). All of them in combination with RBCs initially showed reduction of metHb in HbV, as shown in Figure 8. Among the chemicals tested, AZA, TDB, TLB, and BCB showed similar metHb reduction to that of MB^+. However, PM tended to return to a higher metHb level and TA showed insufficient metHb reduction. A variety of candidate chemicals as electron mediators are known that might differ not only in redox potential, but also in hydrophobicity for biomembrane permeability and reactivity to metHb and molecular O_2. Optimizing metHb reduction is expected to be a challenging study in terms of not only efficacy but also safety. Actually, Kiese et al.[57] reported that TDB is less toxic than MB^+ for the treatment of methemoglobinemic patients. Further study must be conducted to overcome the limitations of this MB^+ system. The reduced MBH would transfer an electron to molecular O_2, and induce reactive oxygen species that are believed to be mostly scavenged by superoxide dismutase and catalase present in RBC. Even so, the generation of reactive oxygen species is expected to be a reason that the metHb level in HbV did not fall below 10%.

In spite of such limitations, we confirmed through in vivo experimentation that coinjection of MB^+ and HbV from the beginning exhibited a lower rate of autoxidation. Late injection of MB^+ at 5 h after single-HbV injection exhibited a rapid reduction of metHb in HbV, as shown in Figure 7. Periodic repeated injection would decrease metHb repeatedly. Actually, our other study showed that three periodic injections of MB^+ at 7, 24, and 48 h after HbV administration maintained a low metHb level for over 50 h. They prolonged the oxygen carrying capacity of HbV. This will be reported elsewhere.

CONCLUSION

We established a system to extract electron energies of RBCs for extracellular chemical reaction using an electron mediator that shuttles across the bilayer membrane. Using this method, reduction of metHb, which is shielded by the liposomal membrane, was performed effectively. Other than HBOCs, our research results imply the possibility of utilizing intracellular abundant glycolytic energy for other electrochemical reactions for in vivo devices.

MATERIALS AND METHODS

Preparation of Hb-Vesicles (HbVs). HbVs were prepared using a method reported previously with only slight modifications.[20,21,43] Human Hb solution was obtained through purification of outdated RBCs provided by the Japanese Red Cross Society (Tokyo, Japan). First, Hb was stabilized using carbonylation (HbCO) and pasteurized (60 °C for 12 h) for virus inactivation. All unstable enzymes were eliminated during this procedure. The obtained Hb solution was concentrated by ultrafiltration to 42 g/dL. Subsequently, pyridoxal 5′-phosphate (PLP; Sigma Chemical Co., St. Louis, MO) was added to the HbCO solution as an allosteric effector at a molar ratio of PLP/Hb tetramer = 1. Here PLP was used instead of 2,3-diphosphoglyceric acid (2,3-DPG) because 2,3-DPG is chemically unstable. The Hb solution with PLP was then mixed with lipids and encapsulated in vesicles. The lipid bilayer comprised 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (Nippon Fine Chemical Co. Ltd., Osaka, Japan), and 1,2-distearoyl-*sn*-glycerol-3-phosphatidylethanolamine-*N*-PEG_{5000} (NOF Corp., Tokyo, Japan) at the molar composition of 5/4/0.9/0.03. The encapsulated HbCO was converted to HbO_2 by exposing the liquid membranes of HbVs to visible light under an O_2 atmosphere. Finally the Hb concentration of the suspension

was adjusted to 10 g/dL. The particle size distribution, 280 ± 50 nm, was measured using a light-scattering method (Nanoparticle analyzer, Model SZ-100; Horiba, Ltd., Kyoto, Japan). The original HbV had metHb level of less than 5%. HbV was incubated at 37 °C for about 20 h to increase the metHb level to about 50%.

Injection of HbV and MB^+ into Wistar Rats. All experimental protocols were approved by the IACUC of Biological Resource Center, A*STAR, Singapore, and Nara Medical University, Japan. The protocols comply with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council − National Academy of Sciences (Washington, DC: National Academy Press, 1996). Experiments were conducted using 21 male Wistar rats (8−10 wks 300−350 g b.w.). The rats were anesthetized by inhalation of 1.5%-isoflurane (Halocarbon Products Corp., North Augusta, SC, USA) mixed with air using a vaporizer (TK-7; Biomachinery, Chiba, Japan) throughout the experiment (fraction of inspired O_2, FiO_2 = 21%; flow rate, 1.5 L/min) while spontaneous breathing was maintained. Polyethylene catheters (SP-31 tubing, OD 0.8 mm, ID 0.5 mm; Natsume Seisakusho Co., Ltd., Tokyo) filled with saline solution containing 10 IU/mL heparin (B. Braun Medical Industries S/B, Penang, Malaysia) were introduced through the left femoral artery and vein. The arterial catheter was connected to a transducer connected to a BP Amp (MLT0670; AD Instruments) and monitored with software (PowerLab; AD Instruments).

Pilot Study. The systemic blood volume was estimated as 56 mL/kg body weight.[13] HbV with the metHb level of about 50% was injected intravenously (10 mL/kg, 1 mL/min). Five minutes later, an MB^+ solution (Sigma-Aldrich; dissolved in PBS at 0.3 mM) was injected (3.3, 6.6, 10 mL/kg, respectively, corresponding to 0.42, 0.83, and 1.27 mg MB^+ /kg). The rat was kept anesthetized for over 5 h for periodical blood collection from the artery. After the experiment, the rat was euthanized by phlebotomy.

Arterial blood (<75 μL) was periodically collected directly into a heparinized glass capillary for hematocrit measurement (Hirschmann Laborgerate GmbH & Co. KG, Eberstadt, Germany). It was centrifuged for 1 min at 12,000 rpm using a centrifuge machine (3220; Kubota Corp., Tokyo, Japan). RBCs precipitated at the bottom, whereas HbV was dispersed in the upper plasma phase. About 20 μL of the plasma phase was diluted with 3 mL of PBS in a Thunberg cuvette that was sealed with a rubber cap. The solution was then deoxygenated using gentle N_2 gas bubbling for 5 min. Consequently, HbV contained only metHb and deoxyHb. Visible light absorption spectra were measured using a spectrophotometer (V-650; Jasco Corp., Tokyo, Japan) that was equipped with an integral sphere (ISN-470) to minimize the effect of light scattering. The spectra were measured between 300 and 500 nm, and the level of metHb was calculated from the absorption ratios at 405 nm (metHb) and 430 nm (deoxyHb).

Practical Injections of HbV and MB^+. HbV with the metHb level below 5% was injected (10 mL/kg, 1 mL/min). The first group received coinjection of MB^+ and HbV. MB^+ was injected 5 min after injection of HbV (10 mL/kg, corresponded to 1.27 mg MB^+/kg). The second group received MB^+ 300 min after HbV injection. The third group received HbV only. The level of metHb was monitored for 7 h using the same method as that described above.

In Vitro Reduction of metHb in HbV in the Presence of Blood Components and MB^+. Heparinized fresh blood was collected from the femoral artery or posterior vena cava of an anesthetized Wistar rat. The blood was centrifuged (2000 rpm, 10 min). Thereby a transparent plasma layer was obtained. After removing the buffy coat (white cells and platelets), the sedimented RBC layer was dispersed in the same volume of phosphate buffered saline (PBS, pH 7.4; Gibco, Paisley, UK). This procedure was repeated twice. The obtained washed RBC concentrate was finally suspended in PBS at [Hb] = 12 g/dL. Whole blood, washed RBC, or plasma was mixed with HbV of about 50% metHb content and incubated at 37 °C. After 5 min, MB^+ (0.3 mM) was added and mixed gently. The change of metHb level of HbV was measured spectrophotometrically as described above. To clarify the origin of electrons, α-D-glucose (100 mM in PBS; Aldrich) as a starting material of glycolysis in RBCs was added at the beginning or at 150 min to the concentration of 10 mM.

For the mixture of washed RBC with HbV and MB^+ at the volume ratio of 2:1:1, the levels of NAD^+, NADH, $NADP^+$, and NADPH in RBCs were monitored using NADP/NADPH and NAD/NADH Quantitation Kits (PromoKine; PromoCell GmbH, Heidelberg, Germany). The procedure followed the instruction manual, except that the RBC concentrate (20 μL) was diluted in an extraction buffer and filtered through 10 kDa centrifugal filter (Ultracel 10K; Amicon Inc.) to remove Hb for the colorimetric assay. The ratios of NAD(P)H to the sum of $NAD(P)^+$ and NAD(P)H were calculated.

Partition Experiment of MB^+ in HbV and RBC. Appropriate volumes of HbV ([Hb] = 3 g/dL) and MB^+ (0.34 mM) were mixed in a tube (MB^+/HbV volume ratio = 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, and 2.0; total 1.5 mL), incubated at 37 °C for 40 min, and ultracentrifuged at 40,000*g* for 1 h. The amount of MB^+ in the supernatant was calculated from the weight of the supernatant and the MB^+ concentration obtained from the absorbance at 665 nm. From the value, the percentage of MB^+ adhering to HbV was determined. The method was repeated for rat RBCs.

In Vitro MB^+ Reduction Using Electron Donors. Reaction profiles of MB^+ and a series of electron donors were tested in anaerobic condition: L-glutathione (GSH; Sigma-Aldrich), L-cysteine (Cys; Kanto Chemical Co. Inc., Tokyo), L-(+)-ascorbic acid (AsA; Wako Pure Chemical Industries Ltd., Osaka, Japan), NADH (Sigma-Aldrich), and NADPH (Sigma-Aldrich). The MB^+ solution dissolved in PBS was mixed with a solution of one electron donor at [MB^+] = 6.8 μM and [electron donor] = 1000 μM in a Thunberg cuvette. For attaining the anaerobic condition, the MB^+ solution in the cuvette was bubbled with N_2 gas beforehand for 10 min. The solution of an electron donor in another glass vial was bubbled with N_2, and collected carefully with a gastight syringe and inserted into the cuvette. Absorption spectra of MB^+ were measured using the spectrophotometer between 500−800 nm. The reduction was monitored at the peak wavelength of 665 nm that corresponds to MB^+.

Liposome Encapsulated NADPH or NADH as a Model of RBCs. Two different powdered mixed lipids were used for liposome formation. One is the same with the lipids of HbV (DPPC/cholesterol/DHSG/DSPE-PEG_{5000} = 5/4/0.9/0.03 by mol). The other includes 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC; Nippon Fine Chemical Co. Ltd.) instead of DPPC (DMPC/cholesterol/DHSG/DSPE-PEG_{5000} = 5/4/0.9/0.03 by mol). NADH or NADPH was dissolved in PBS at

the concentration of 25 mg/mL. The powdered mixed lipid (0.3 g) was dispersed in 5 mL of NADH or NADPH solution and stirred overnight for encapsulation. The particle diameter was regulated using the stepwise extrusion method with the final filter of 0.22 μm pore size (FM; Millipore). The unencapsulated outer NADPH or NADH was removed using a gel filtration using Sepharose CL-4B (Sigma Chemical Corp.). On the gel filtration column (i.d. 1.5 cm; length 18 cm), 1 mL of the liposome suspension was embedded. The filtrated liposome fraction was obtained. Then the whole lipid concentration was adjusted to about 0.6−0.7 g/dL. The liposome suspension was mixed with HbV with 50% metHb level and incubated at 37 °C in anaerobic condition. After 5 min MB^+ was injected to initiate electron transfer. The volume ratio of NAD(P)H-encapsulated liposome:HbV:MB^+ was 10:1:1. The surfaces of both the NAD(P)H-encapsulated liposomes and HbV are PEG-modified. The change of metHb level was monitored spectrophotometrically as described above. We confirmed that the liposomes did not release NAD(P)H during the procedure.

Other Electron Mediators for metHb Reduction in HbV. Instead of MB^+, other electron mediators were tested for metHb reduction, including Azure A (AZA; MP Biomedicals), phenazine methosulfate (PM; MP Biomedicals, Santa Ana, USA), thionin acetate (TA; Wako Pure Chemical Industries Ltd.), toluidine blue O (TDB; Waldeck GmbH & Co., Munster, Germany), Brilliant cresyl blue (BCB; MP Biomedicals), and toluylene blue (TLB; MP Biomedicals). They were dissolved at 0.35 mM in PBS without further purification. The washed rat RBC suspension ([Hb] = 12 g/dL), HbV with 50% metHb level, and a solution of an electron mediator were mixed at the volume ratio of 5.6:1:1. The change of metHb level in HbV was monitored using the method described above.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): Of the authors, H.S. is an inventor holding patents related to the production and utilization of Hb-vesicles.

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