Chapter 5

Artificial Red Blood Cell As a New Therapeutic Tool for Coronary and Cerebral Ischemia: s-Nitrosylated Polyethylene Glycol-conjugated Hemoglobin, a Promising Candidate

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1. INTRODUCTION

Several hemoglobin (Hb)-based derivatives have been studied for use as artificial oxygen carriers. However, they have several limitations[1]. The major side effects include vasoconstriction, abnormal gastrointestinal constriction and platelet stimulation. The most plausible mechanism for these side effects is the nitric oxide (NO) scavenging by acellular Hb itself, since the heme of Hb has a high affinity to NO. A universal problem among all Hb derivatives is their short plasma residence time. The half life time of Hb derivatives in the circulation ranges from 6 to 24 h in animals, and these values are very much shorter than the mean residence time of 120 days for human red blood cells. The hope, therefore, is that the new products will have better applicability in clinical situations where the short-term use of an oxygen carrier is essential.

Recently, it was proposed that the Cysβ93 of Hb is covalently bound with NO, and that this s-nitrosylated Hb (SNO-Hb) retains EDRF/NO-like bioactivity[2), 3)]. Since s-nitrosothiols do not react with heme of Hb, SNO-Hb can provide a protected way of delivery of bioactive NO to the tissues. Indeed, SNO-Hb induces relaxation of pre-capillary vessels and inhibits platelet aggregation[3), 4)]. These insights suggest that SNO-Hb can release NO preferentially where pO_2 is low, dilating small vessels, thus providing more blood to the ischemic tissues. In the circulation, SNO-Hb may still have vasoconstrictive activity because Fe(II)-Hb, which binds oxygen, also has an affinity to NO, though SNO-Hb can compensate for this vasoconstricting effect by releasing NO. These dual functions as both a scavenger and donor of NO not only contribute to avoidance of Hb-induced vasoconstriction, but increase the therapeutic potential of SNO-Hb for use in the area of oxygen therapeutics.

2. S-NITROSOHEMOGLOBIN

Hb is a tetramer composed of two α- and two β-subunits. In human Hb, the β-subunit contains one highly reactive sulfhydryl group (Cysβ93). This sulfhydryl residue has been reported to be s-nitrosylated to form SNO-Hb within red blood cells[2)]. The authors showed a dynamic cycle in which the binding of oxygen to heme iron promotes the binding of NO to the sulfhydryl residues, and deoxygenation is accompanied by an allosteric conformational change that releases the NO group. In this context, Hb is *s*-nitrosylated in the lung when red blood cells are

oxygenated, and the NO group is released during arterial-venous transit dependent on the oxygen gradient in the tissues[3]. SNO-Hb, therefore, can release an NO-group to induce vasorelaxation and increase regional blood flow, and then deliver oxygen more efficiently to the tissues with oxygen requirements.

2.1 Preparation of SNO-PEG-Hb

To utilize SNO-Hb as an oxygen carrier, we have developed a pyridoxalated and pegylated SNO-Hb derivative having low oxygen affinity and an optimum plasma residence time. The preparation steps are schematically illustrated in Figure 1. The detailed preparation method is described elsewhere[5]. Briefly, human Hb purified from outdated human red cell products was mixed with pyridoxal-5' phosphate and pyridoxalation was started by the addition of sodium borohydrate under anaerobic conditions. For the pegylation of pyridoxalated Hb, the activated ester of PEG-bis (succinimidyl succinate) was added very slowly with stirring. *S*-nitrosylation of PEG-Hb was then performed with addition of s-nitrosoglutathione. The yield of s-nitrosylation was estimated by using a high-performance liquid chromatography (HPLC) coupled with flow reactors of metal and Griess reagent (Fig. 2)[6]. In human Hb, Cysβ93 is highly reactive and the prefered target for s-nitrosylation. The content of NO in SNO-Hb reported in the text was, therefore, expressed on the basis that a fully *s*-nitrosylated Hb (100% SNO-Hb) contains two NOs because one tetramic Hb is constituted from two β-subunits. The yield of *s*-nitrosylation was usually set to 30-37%.

3. EFFICACY AND SAFETY OF SNO-PEG-HB

3.1 Vasoactivity of SNO-PEG-Hb

Systemic administration of unmodified Hb resulted in a hypertensive reaction, which result is in agreement with previous reports[7, 8]. In contrast, SNO-Hb did not raise blood pressure, suggesting that NO released from SNO-Hb may have compensated for NO scavenging by the heme in SNO-Hb. Since PEG-Hb also caused no significant increase of blood pressure, PEG modification itself may also contribute to avoidance of Hb-induced hypertension, probably because the

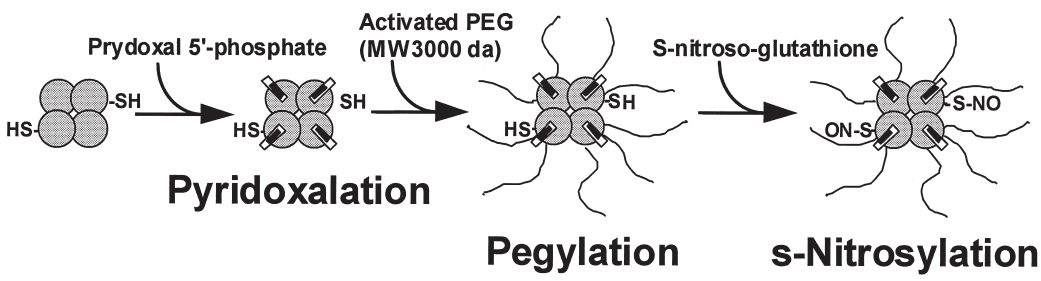

Figure 1 SNO-PEG-Hb preparation. Hb was pyridoxalated, pegylated and then *s*-nitrosylated.

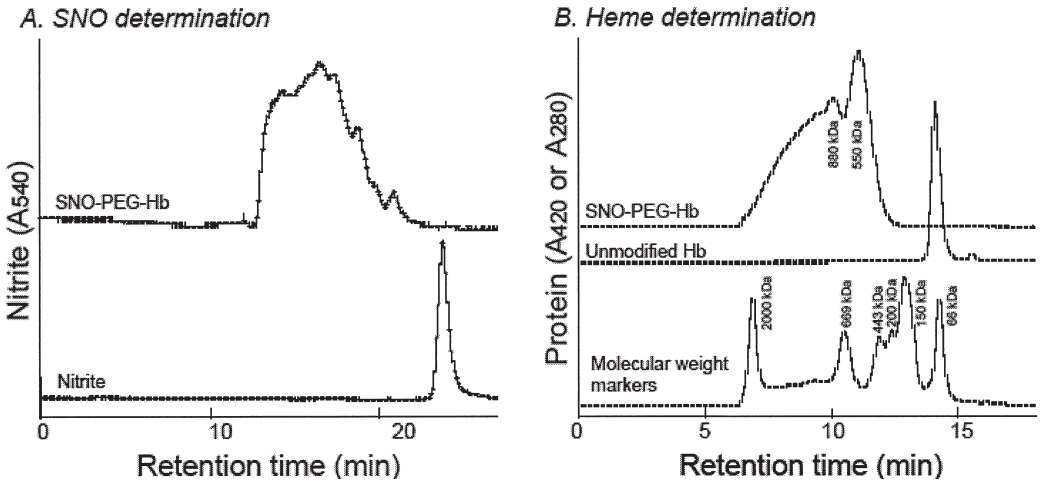

Figure 2 HPLC characterization of (A) Hb-bound NO and (B) heme of SNO-PEG-Hb. (A) Samples were separated on a gel-filtration column (8 x 300 mm, GFC-200, Eicom, Kyoto, Japan) eluted with 10 mM acetate buffer, 0.1 mM EDTA, 100 mM sodium chloride, pH 5.5, at the flow rate of 0.55 mL/min. The eluate was mixed with 1.75 mM mercury chloride at the flow rate of 0.20 mL/min to decompose *s*-nitrosylated protein, and further mixed with Griess reagent at the flow rate of 0.22 mL/min. The red azo-dye formed was determined by the absorption at 540 nm. (B) For the characterization of molecular weight distribution, proteins were separated on a gelfiltration column (7.6 x 300 mm, TSK G3000SW, Toyo Soda Co. Ltd, Tokyo, Japan) in 10 mM sodium phosphate buffer, 100 mM sodium chloride, pH 6.9, at the flow rate of 0.9 mL/min. Proteins were monitored at 420 nm for heme and at 280 nm for molecular weight markers.

extravasation of Hb molecules and the resultant NO scavenging in the vessel walls is critical for Hb-induced vasoconstriction[9]. Pegylation effectively prevents the extravasation of Hb molecules. SNO-PEG-Hb showed reduced hypertensive activity like that of SNO-Hb (Fig. 3).

The release of NO from SNO-Hb was reported to be accelerated in the presence of low molecular weight thiols such as glutathione and a trace amount of copper ions[6]. Since both components should be present in blood plasma, we assumed that the half-life of NO bound to PEG-Hb in the plasma might be very short. We measured the half-life of Hb-bound NO as shown in Figure 4. The present data indicated that the plasma residence time was not so short. One possible reason for this might be the very low concentration of free copper ion in plasma. Finally, these findings suggested that SNO-PEG-Hb was a slow-releasing agent for NO.

3.2 Oxygen Transporting Capacity

The oxygen transporting capacity of SNO-PEG-Hb was evaluated using a hemorrhagic shock model in rats by monitoring the redox state of cytochrome oxidase reduction of cerebral tissues, in which a near-infrared spectroscopy was

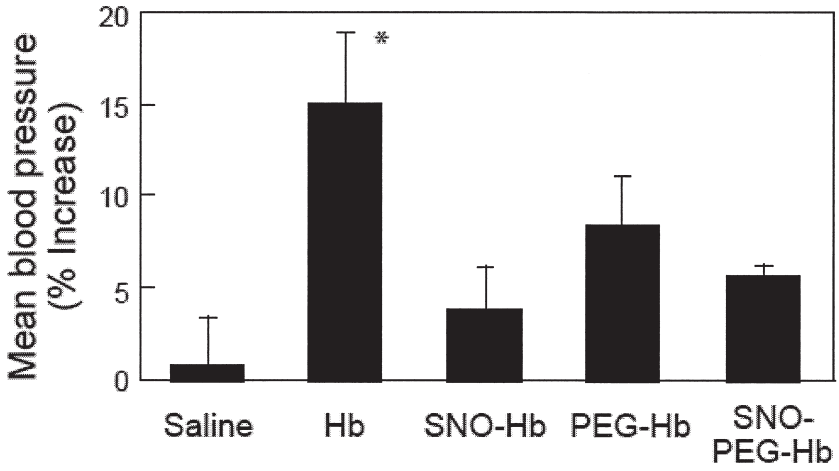

Figure 3 Changes in mean arterial blood pressure 5 min after a bolus injection of Hb materials (125 mg Hb/kg, Hb 10% solution) into male Wistar rats. Relative increase was calculated from the data before and after the injection. Each value represents the mean±SEM of 6-9 animals. *$p < 0.05$ vs saline control by ANOVA followed by Scheffe's test.

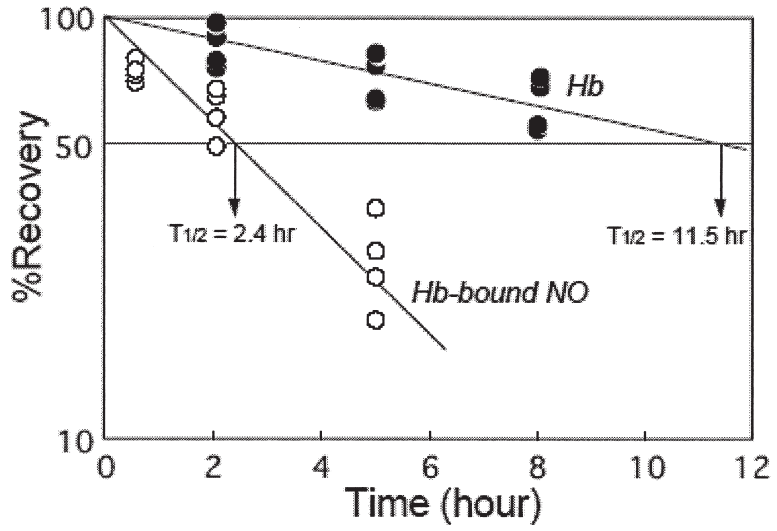

Figure 4 Plasma retention of NO (open circles) and heme (closed circles) of SNO-PEG-Hb in the circulation of rats after a bolus injection at 125 mg/kg.

used[10]. After blood was removed (up to 30%) from anesthetized male Wistar rats under 21% O_2 ventilation, saline, PEG-Hb or SNO-PEG-Hb (5% Hb solution) was isovolumetrically infused. The pO_2 in the cervical vein and arterial blood pressure were monitored throughout the experiments. The intravenous infusion of SNO-PEG-Hb and PEG-Hb restored oxy-Hb, total-Hb, and cytochrome oxidase reduction

levels of cerebral tissues (Fig. 5). This suggested that SNO-PEG-Hb could supply enough oxygen to the brain, like PEG-Hb. During the Hb infusion, PEG-Hb could quickly restore blood pressure, while this recovery was slower after the infusion of SNO-PEG-Hb. This suggested that SNO-PEG-Hb causes vasodilation as an NO donor.

3.3. Safety Characteristics

Safety evaluation of SNO-PEG-Hb was performed in a volume overload experimental model using rats. Unmodified Hb, PEG-Hb and SNO-PEG-Hb were infused into male Wistar rats at 1.15 g/kg body weight (5% Hb solution). Bovine serum

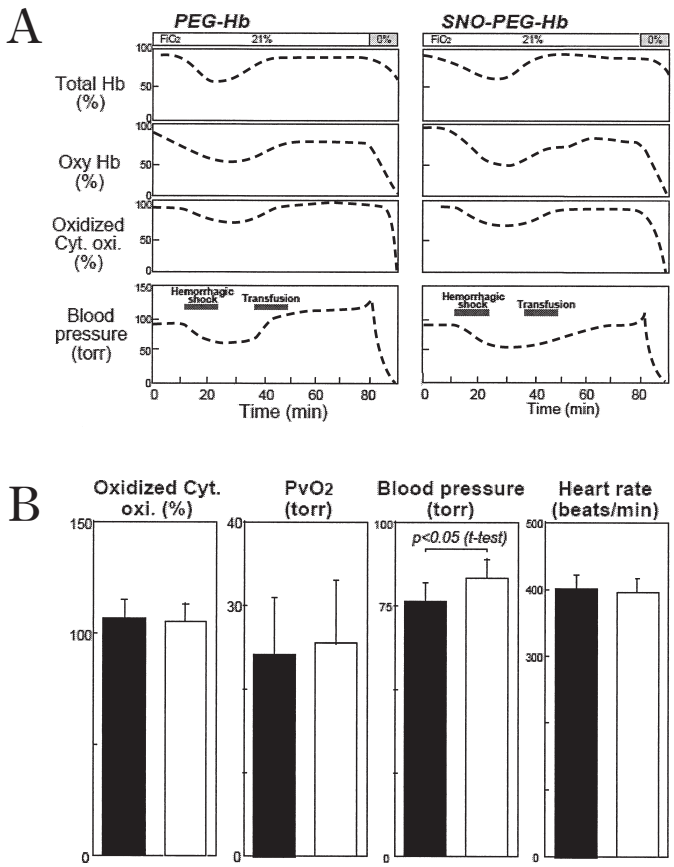

Figure 5 The redox status of cytochrome oxidase in the rat brain after hemorrhagic shock and Hb infusion. (A) Typical tracings; (B) Comparison at the end of Hb infusion. The duration of hemorrhage and transfusion was indicated by bars. The relative change against the value obtained during the resting period is indicated in some figures.

albumin was used as the control. Blood samples were obtained 1, 3, 7 and 14 days after administration. Plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrigen (BUN) and creatinine were assayed, and the liver and kidney were removed, fixed with formaline and stained for histopathological examination. All protein solutions induced transient increases in AST and ALT, and a decrease in BUN (Fig. 6). The most remarkable increase in ALT was observed with PEG-Hb, while SNO-PEG-Hb showed the smallest increase. These changes in AST and ALT returned to the baseline at 3 days postinfusion. Histological examinations of the liver supported these biochemical observations (Table 1). In kidneys, unmodified Hb caused red casts immediately after the administration and increased the plasma creatinine level at 7 days postinfusion, suggesting that unmodified Hb was nephrotoxic. PEG-Hb and SNO-PEG-Hb caused vacuole formation in the proximal tubular epithelium. Cellular infiltration in the interstitium was observed in the PEG-Hb group, while this change was rare in the SNO-PEG-Hb group. Renal function, as evaluated by BUN and creatinine, was, however, normal in both Hb groups. These findings suggested that two pegylated Hb derivatives induced some transient stress with regard to hepatic

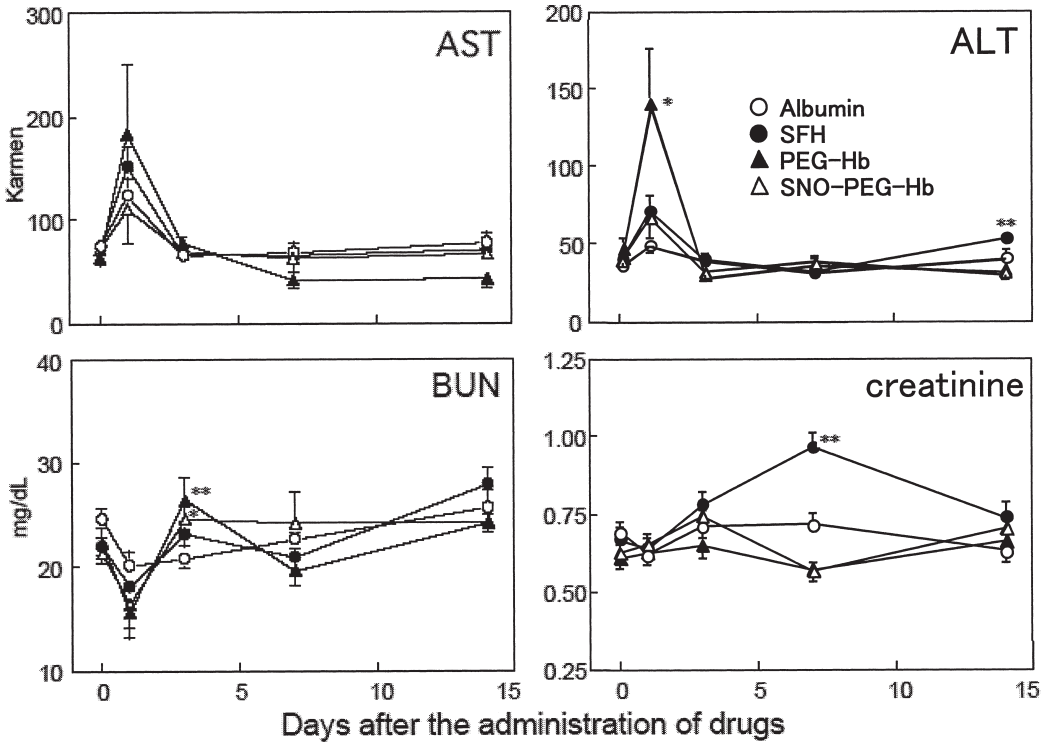

Figure 6 Changes in AST, ALT, BUN and creatinine after the administration of Hb solutions (1.15 g/kg, Hb 5% solution) into male Wistar rats. Each value represents the mean ± SEM of 7-13 animals. *$p<0.05$ and **$p<0.01$ vs albumin control by ANOVA followed by the Fisher PLSD test.

Table 1 Histopathological observation of the liver and kidney 7 days after the Hb administration into rats

Drugs	Albumin	Hb	PEG-Hb	SPEG-Hb
Number of animals observed	8	8	8	9
Liver				
Focal capsulitis	4	3	5	3
Focal fibrous thickening of the capsule	3	3	5	4
Granuloma in the capsule	0	0	1	0
Focal necrosis	0	0	0	0
Fatty droplets in the hepatocytes	0	0	1	0
Kidney				
Vacuolization of the proximal tubular epithelium	0	0	8	8
Red (hemoglobinogenous) casts	0	2	0	0
Focal basophilic changes and atrophy of the renal tubules	1	2	1	1
Cellular infiltration in the interstitium	0	0	1	0
Pyelitis	0	0	1	0

and renal functions, but it seemed to be well tolerated.

The most remarkable observation in the histology was the vacuolization in the proximal tubular epithelium of the kidneys in the animals transfused with the two pegylated Hb products (Color Fig. 17 for histology of rats treated with SNO-PEG-Hb). A similar phenomenon has been reported by Matsushita et al.[11], who showed that a PEG-Hb product caused vacuole formation in the epithelium of the canine kidney, without any pathological features of renal ischemic changes and the regeneration of the tubules. They demonstrated that these vacuoles included iron, possibly derived from the Hb product, based on the data of elemental X-ray microanalysis and ferric iron staining. Other substances such as dextran, hydroxyethyl starch, sucrose and mannitol have been also shown to cause vacuolization without any disturbance in renal functions. These reports suggest that the vacuolization reflects the degradation process of artificial materials in the proximal tubule cells.

4. IMPLICATION OF S-NITROSOTHIOLS

There is increased interest in NO in the body as a result of its formation by a variety of cell types as endothelial cells, platelets, neutrophils, and smooth muscle cells. The redox and chemical states of NO are critical in the diverse physiological and pathophysiological events induced by NO. One mechanism by which NO alters the biological function is through formation of s-nitrosothiols; there is increasing evidence that s-nitrosylation plays a large role in regulation of key enzymes and transcription factors. For example, the cardiac calcium release channel (ryanodine receptor) has been shown to be reversibly regulated by *s*-nitrosylation[12]. *S*-nitrosylation of α_1-protease inhibitor (αPI), a major serine protease inhibitor protein in human plasma, gives a novel function to the protein; SNO-αPI exhibits

remarkable cytoprotective effects in ischemia-reperfusion injury[13]. NF-kB is one of several transcription factors that display redox-sensitive DNA binding, and *s*-nitrosylation of its redox-sensitive cysteine-62 residue has been shown to inhibit NF-kB-dependent transcription[14]. Recently, several active transporting systems of RNSO-bound NO into live cells beyond the cell membrane have been reported[15), 16]. The specific enzyme for GSNO degradation has been also identified as glutathione-dependent formaldehyde dehydrogenase, and this enzyme is evolutionally conserved from bacteria to humans[17]. Even though the total picture of the biology of RSNO is still unknown, these findings suggest the presence of an active regulatory system for RSNO metabolism, and that RSNO-bound NO may play essential roles in NO biology in health and disease.

5. FUTURE PERSPECTIVES OF SNO-PEG-HB

Since SNO-PEG-Hb may share properties similar to those of SNO-Hb, SNO-PEG-Hb is expected to provide oxygen and NO to the tissue where oxygen tension is low, which results in an increase in regional blood flow. Furthermore, regionally released NO may contribute to regulate the above key enzymes and transcription factors. Moreover, SNO-PEG-Hb is also able to quench NO where it is produced in excess amount such as inflammation and ischemia reperfusion. We are now trying to prove the neuroprotective effects of SNO-PEG-Hb after cerebral or cardiac transient ischemia. In such circumstances, not only NO-quenching effects of SNO-PEG-Hb, not also its oxygen delivering properties will act in favor of ischemic regions. Considering that SNO-PEG-Hb may be applied to such physiopathological situations, further characterization of SNO-PEG-Hb as a therapeutic tool should be awaited.

6. CONCLUSION

We have developed a new Hb derivative that can deliver and release oxygen and NO-group molecules in the periphery. This product might be a valuable artificial oxygen carrier that can be used as a tool for oxygen therapeutics against cardiac and cerebral ischemia.

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