maintenance of vascular tone. However, increased intracellular concentration of active oxygen species inactivates NO, and inhibits the production of prostacyclin in endothelial cells by inhibiting the arachidonic acid cascade. Higher levels of active oxygen species result in the destruction of the enzymes, with the exception of thromboxane A_2 synthetase that is resistant to inhibition and destruction by free radicals. The net effect of these interactions is vasoconstriction [48,49]. Moreover, active oxygen species generates peroxides, which will also deteriorate the enzyme activity.

A common condition to Hb-based red cell substitutes is the relatively rapid metHb formation in the circulation. It is well known that HbO_2 dissociates into metHb and O_2^-. Although the direct relationship between vasoconstriction and O_2^- generation from red cell substitutes has not been well established, suppression of metHb formation may be important not only to maintain the function but also to reduce the generation of superoxide which may act as a vasoconstrictor. In RBCs metHb reduction system includes cytochrome b_5, NADPH-flavin, direct reduction by glutathione, and ascorbic acid, and scavengers for active oxygen species such as superoxide dismutase for O_2^- and catalase for H_2O_2. Preservation of the native enzyme activities is one method of reducing generation of metHb and active oxygen species [46]. However, during the purification of Hb especially at the heat sterilization, the enzymes are completely removed. In the case of acellular Hb, direct conjugation of catalase and superoxide dismutase has been studied to suppresses metHb formation [50]. For cellular HbV, preservation of native enzymatic systems [46] or encapsulation of a certain amount of reductants and the enzymes have been proposed as a method for suppressing metHb formation [51], which may be effective to reduce active oxygen species generation and vasoactivity.

Complement activation and vasoactivity

Anaphylatoxins are pharmacologically active peptides formed in blood during enzymatic activation of the complement system by the presence of foreign elements or inflammation. C3a, a peptide derived from the third component of complement, stimulates smooth muscle contraction, releases histamine from mast cells and increases vascular permeability. Topical applications of C3a and histamine induce vasoconstriction of feeding arterioles [52], and the anaphylatoxin C5a stimulates platelet activity and arachidonic acid cascade releasing thromboxane A_2, which is a strong vasoconstrictor.

Fluosol-DA and some liposome products have been reported to show anaphylactic reactions with thrombocytopenia and increase blood thromboxane A2 concentration [53,54], though vasoconstriction was not studied. Infusion of prednisolone, indomethacin, or complement receptors is one method to prevent complement activation. Surface modification of liposome-encapsulated hemoglobin with polyethyleneglycol (PEG) is another way [8,55] to prevent access of plasma proteins including complement. The effectiveness of PEG in improving blood rheology was reported by the

groups at Waseda and Terumo, and effectiveness in the reduction of complement activation was reported by the Naval Research group at the 7th ISBS.

Summary

Analysis of the microcirculation is important to evaluate the efficacy of red cell substitutes because this is the site of oxygen exchange. PEG-modified HbV have been developed with stable and uniform characteristics that show improved microvascular responses to severe hemodilution compared with unmodified HbV and albumin alone. There have been many vasoconstrictive factors reported so far in the presence of red cell substitutes, which cannot be explained solely on the basis of nitric oxide-related reactions. Resistance vessels appear to be crucial for the regulation of microvascular flow, and the study of these vessels may be important to improve the efficacy of red cell substitutes.

Acknowledgments

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CHAPTER 19

The Heme Oxygenase System In Liver Microcirculation: A Key Mechanism for Hemoglobin Degradation

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Introduction

Modified hemoglobin derivatives have been developed as blood substitutes clinically applicable to rescue shock states. However, their degrading process in reticuloendothelial system and its pathological consequences remain unknown. Physiological degradation of heme (iron-protoporphyrin IX) into biliverdin, iron and carbon monoxide (CO) is mediated by heme oxygenase (HO), which consists of two distinct isoenzymes called HO-1 [1] and HO-2 [2]. HO-1, also known as the heat shock protein-32 (hsp 32), is induced by a variety of stressors such as hyperthermia [3], cytokines [4], and intake of heavy metals [5]. This isoform is considered to be present in spleen, a major organ for destruction of senescent erythrocytes, and in liver stimulated with endotoxin [6] or ischemia-reperfusion [7]. On the other hand, HO-2 is a constitutive form and is known to be abundant in brain, testis and unstimulated liver of rodents and human, as reported previously [8]. Because of little information as to topographic distribution of the heme oxygenase system, a fate of artificial blood substitutes administered in the body has not fully been investigated at cellular and molecular mechanisms in vivo. This chapter aimed to focus on biological significance of distribution of HO isoforms in hepatic microvascular units as a primary determinant of heme degradation.

Heme oxygenase-1: a primary anti-oxidative defense mechanism

Heme is metabolized to biliverdin and CO by heme oxygenase (Fig. 1). In humans, nearly 80% of the bilirubin excreted in bile is derived from hemoglobin heme [2]. Cytochrome P450 is a major contributor to the bilirubin derived from non-hemoglobin sources. Recent investigation has revealed that the inducible form of heme oxygenase (HO-1) plays an important role in modulation of cell susceptibility to oxidative stress or lipid peroxidation. Namely, the induction of this enzyme can

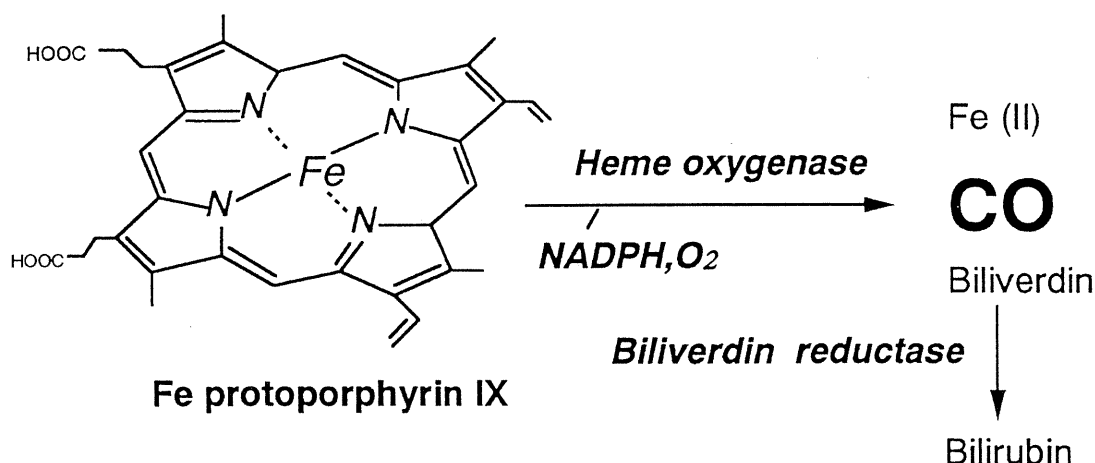

Fig. 1. Heme oxygenase and generation of carbon monoxide and bilirubin.

reduce the intracellular free heme pool and increase bilirubin. Furthermore, the heme oxygenase reaction is known to regulate the expression of ferritin, the iron-storaging protein through the mechanism involving free iron yielded by heme degradation. When skin fibroblasts are exposed to ultraviolet (UV) A, these cells induce ferritin and acquire resistance to the second exposure to the lethal dose of UVA. The UV-induced induction of ferritin and acquisition of anti-oxidant properties can be abolished by inhibiting heme oxygenase or by chelating the iron by desferrioxamine. These results imply the importance of HO-1 as a crutial enzymatic intermediate in an oxidant stress-inducible antioxidant defense mechanism involving the induction of ferritin [9].

Another possibility by which the HO reaction serves as an anti-oxidative defense mechanism can be explained by its involvement in generation of bilirubin, a potent endogenous radical scavenger [10,11]. Bilirubin is generated by the reaction of biliverdin reductase, a terminal step for heme degradation. Because of its capability to scavenge single oxygen and hydroxyl radicals, bilirubin is called "suicide anti-oxidant". It has well been demonstrated that bilirubin endogenously upregulated as a consequence of an induction of HO-1 plays a role in reducing the oxidative impact under disease conditions such as endotoxemia. It has also been shown that bilirubin can attenuate transendothelial migration of macrophages prestimulated with oxidized low-density lipoprotein.

Roles of carbon monoxide derived from heme oxygenase in the liver

The HO reaction generates another biologically important product, that is, CO. Can this gaseous monoxide exert its biological actions to regulate cell and organ function? Since the putative signal transducing mechanism of CO and NO involving soluble guanylate cyclase-cGMP pathway was recently proposed, several lines of

circumstantial evidence have emerged illustrating the mechanisms for CO-dependent regulation of cell function. Such evidence was first raised by Snyder and his coworkers, who showed that endogenous CO suppression by zinc protoporphyrin IX (ZnPP), a competitive inhibitor of the HO reaction, induces reduction of cGMP in olfactory nerve cells which possess little NO synthase in spite of the presence of soluble guanylate cyclase [12]. As shown later, we reported that ZnPP administration to the perfused rat liver raises an increase in the vascular resistance which can be recovered by supplement with micromolar levels of CO [13–15]. In addition, it has recently been shown that the inducible form of heme oxygenase is expressed by vascular smooth muscle cells and CO derived from the enzyme activity determines cGMP levels in these cells, suggesting that the possible role of endogenous CO in autocrine regulation of vascular tone [16].

On the other hand, evidence has recently been provided that, as compared with NO, CO is not such a potent mediator that it cannot activate soluble guanylate cyclase. The ability of NO to activate this enzyme reaction is approximately 50-fold greater than that of CO [17]. Different from NO, CO is a non-radical monoxide, and its reactivity with biological reagents which can interact with NO such as SH compounds or non-heme iron is far less than that of NO. In other words, CO cannot function as a potent activator of guanylate cyclase under circumstances in which the local concentration of NO is at relatively higher levels than that of CO. It is therefore impossible to demonstrate the significance of CO in cGMP-dependent regulation of cell and organ function without discussing the actual amounts of the gaseous mediator in situ and the relationship of microanatomical orientation between the CO-generating effector cells and target cells in each experimental system.

Distribution of heme oxygenase isoforms in liver

Liver is a major organ in the body which continuously generates CO through the HO reaction. Several lines of evidence have indicated that the HO reaction serves as a key mechanism to maintain the integrity of physiological function of organs such as liver. As described previously, zinc protoporphyrin IX (ZnPP), a heme oxygenase inhibitor, elicits a marked increase in the vascular resistance as a consequence of sinusoidal constriction [13,14]. Furthermore, the ZnPP administration turned out to induce bile acid-dependent choleresis which coincided with a depletion of the venous CO flux and biliary excretion of bilirubin, another product of heme degradation [18]. The results have thus suggested that CO generated by heme oxygenase serves as an endogenous regulator of hepatobiliary functions under physiological conditions.

In an attempt to understand the aforementioned roles of heme oxygenase, we have recently investigated the intrahepatic distribution of HO-1 and HO-2 using newly developed monoclonal antibodies (MoAbs) against these isozymes [19]. The immunohistochemical analysis disclosed different topographic patterns in the distribution of the two isozymes. As seen in the left panel of Fig. 2, HO-1 distributed in a relatively

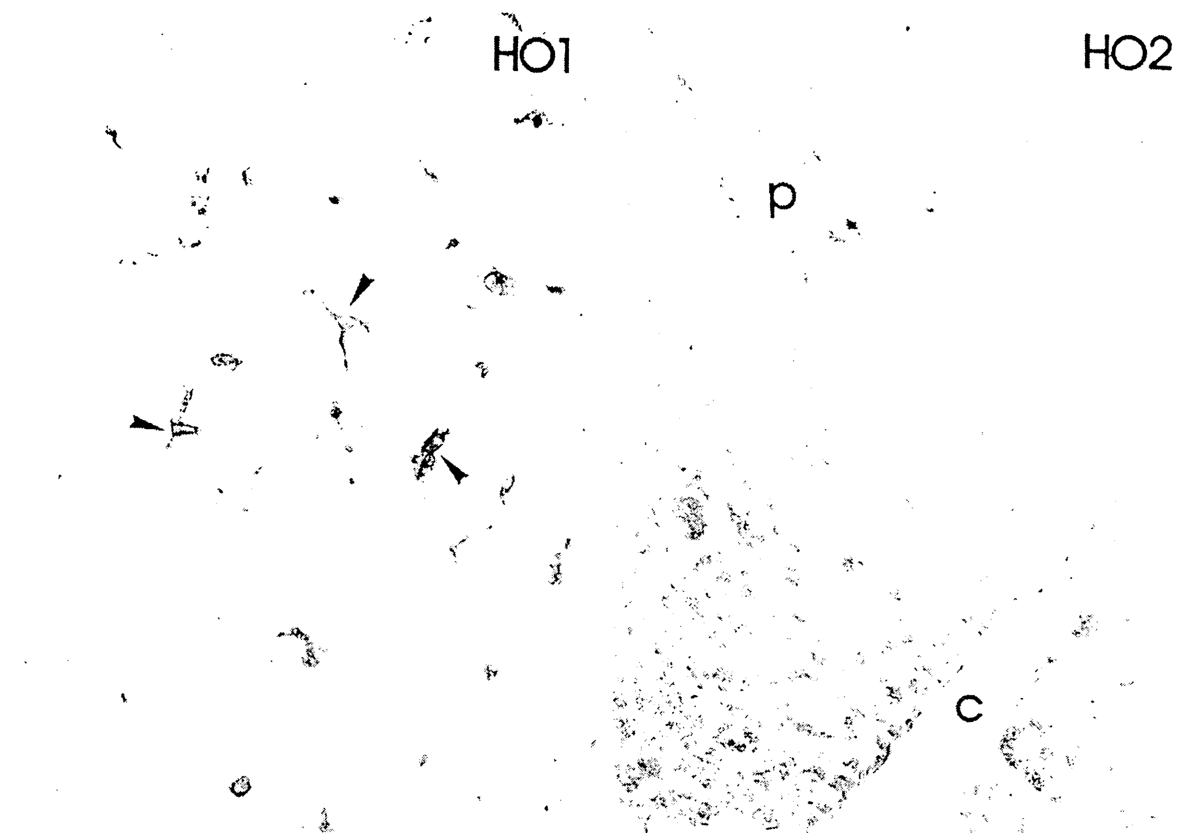

Fig. 2. Expression of HO-1 and HO-2 in the normal rat liver. The 10-μm thick sections were immunostained with MoAbs against HO-1 (left) and HO-2 (right). P and C denote portal and central venules, respectively. Arrowheads suggest that Kupffer cells constitute a major site of the HO-1 expression. Bars represent 50 μm and 100 μm in left and right panels, respectively. Cited from J. Clin. Invest. vol 101, 1998 with permission.

small number of cells which scattered over the entire lobules. These HO-1-positive cells were characterized by their irregular and dendritic shapes and by protrusion of their cytoplasm towards sinusoidal spaces, suggesting the presence of HO-1 in Kupffer cells. On the other hand, hepatocytes exhibited little staining, if any. By contrast, HO-2 occurred in the parenchymal cells and distributed homogeneously among the entire lobules, while nonparenchymal cells displayed practically no staining (right panel in Fig. 2). We further attempted to determine specific cell types that express HO-1 among the nonparenchymal cells, indicating that hepatic stellate cells exhibited little HO-1 staining, if any. These findings indicate that Kupffer cells constitute a major cellular compartment responsible for the intrahepatic HO-1 expression.

The intrahepatic distribution of HO-1 exhibited quite different pictures when the liver was prestimulated with LPS, an inducer of this enzyme [19]. The liver exposed to the 6-h pretreatment revealed that HO-1 not only occurred in tissue macrophages but was also induced markedly in hepatocytes. On the other hand, the HO-2 staining did not display any notable changes as compared with the control liver. These results

indicate that Kupffer cells and hepatocytes constitute major cellular components that express the inducible HO isozyme in the endotoxin-treated liver.

Microtopographic basis for CO-mediated vasorelaxation in liver

Blood substitutes which are going to be applied in the clinical field can not only bind molecular oxygen but also trap vasodilating gaseous monoxides such as NO and CO. Considering that endothelial cells in the hepatic microvessels (sinusoids) possess abundantly small pores called sieve pores or fenestration which allows macromolecules in circulation to access the extrasinusoidal space (the space of Disse) (Fig. 3), it is hypothesized that hepatic microvascular responses may differ between stroma-free hemoglobin (Hb) molecules and liposome-encapsulated Hb (HbV) that cannot access the space of Disse because of its greater size than that of fenestration. We have attempted to examine this hypothesis in isolated perfused liver preparation. As seen in Fig. 4, immediately after the start of HbO_2 administration at a concentration of 1.5 g/dl, the resistance increased markedly, showing a 25% elevation as compared to that in the steady-state conditions. When the perfusate was replaced by the Hb-free buffer at 15 min, the increased resistance decreased gradually and reached the control level at 30 min, indicating that the HbO_2-induced vascular response is reversible. It

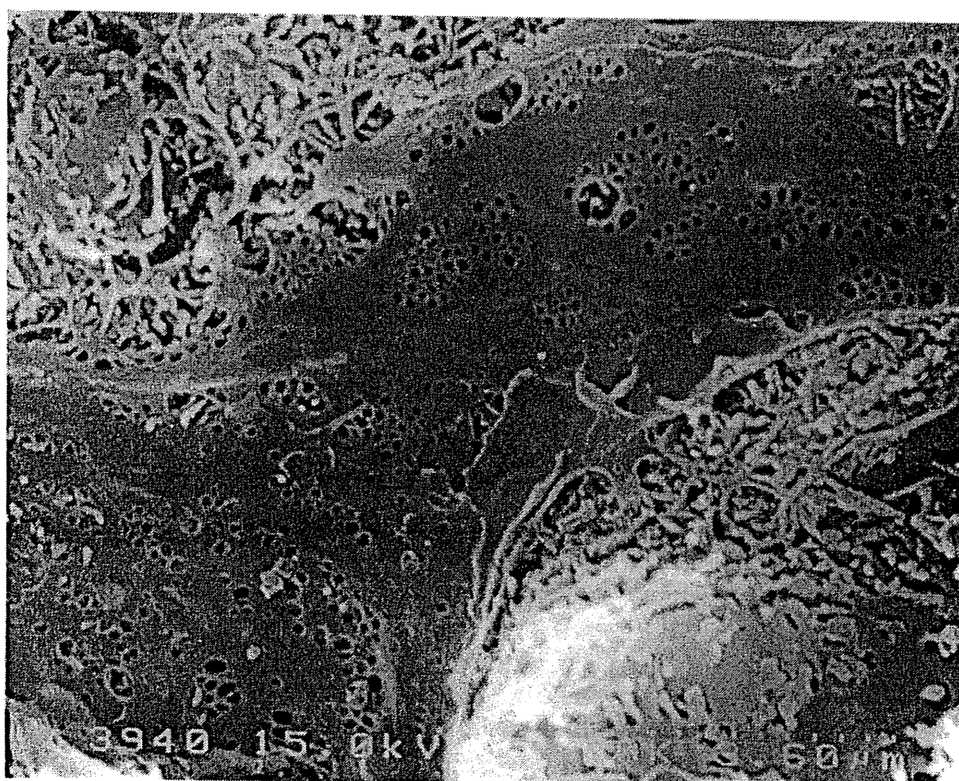

Fig. 3. A representative scanning electron micrograph showing sieve pores in sinusoidal endothelial cells in the rat liver. Size of pores are in a range between 100–150 nm in diameter.

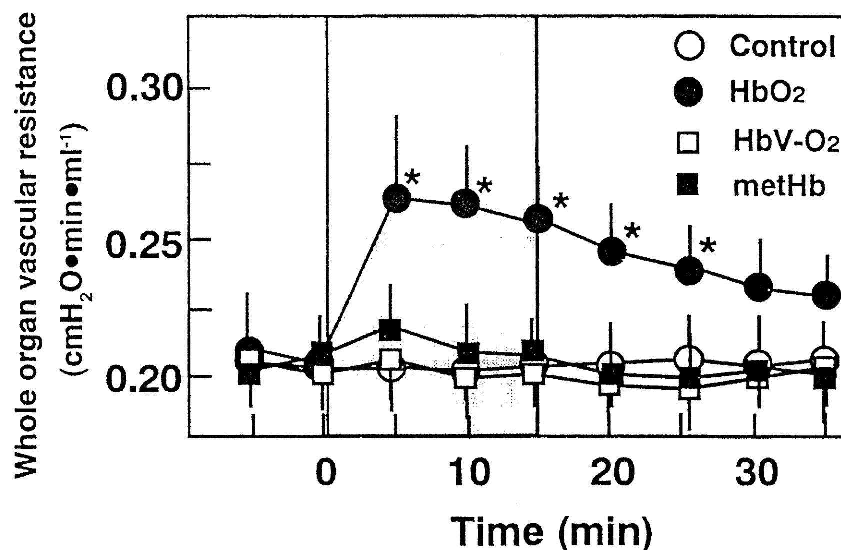

Fig. 4. Time history showing effects of Hb derivatives on the whole organ vascular resistance in perfused rat liver. The Hb derivatives (HbO_2, HbV-O2, metHb) were administered at a final Hb concentration of 1.5 g/dl for 15 min as seen in the shaded area. Data represent mean ± SE of 6 experiments in each groups. Open and closed circles indicate the data collected from the control and HbO_2-treated livers, respectively. Shaded circles and open squares are those collected from the groups treated with HbV-O_2 and metHb, respectively. $^*P < 0.05$ as compared with the data in the control group. Cited from J. Clin. Invest. vol 101, 1998 with permission.

should be noted that the HbV-O_2 administration did not evoke any significant elevation in the resistance. These results suggest that the HbO_2-induced elevation of the vascular resistance was abolished when the diffusion of Hb across the sinusoidal endothelium into the space of Disse was blocked by its liposomal encapsulation. In addition to these findings, metHb, a reagent that could scavenge NO but not CO, did not alter the vascular resistance, suggesting that CO plays a major role in lowering the vascular resistance under the current experimental conditions. Furthermore, under these experimental conditions, inhibitors of NO synthase such as *N*-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine did not alter the basal vascular resistance. These results suggest that involvement of NO is little, if any. Taking these circumstances into account, we suggest that, once administered, stroma-free HbO_2 diffusing into the space of Disse eliminates CO in situ and thereby evoking sinusoidal constriction.

Design of blood substitutes based upon heme oxygenase biology

When the previously proposed mechanisms for intrahepatic heme degradation are taken into accounts, the present findings suggest a possible cooperative role of these two isoforms in catabolism of hemoglobin-derived heme in different cellular

compartments. Namely, previous studies by Bissel et al. [20] revealed that removal of senescent erythrocytes from the circulation is carried out by macrophages in the liver and spleen, while hemoglobin released as a consequence of erythrocyte destruction can be metabolized mainly in the liver parenchyma. Spontaneous expression of the inducible HO isoform in Kupffer cells of the control liver appears to result from constant exposure of the cells to senescent erythrocytes in the sinusoidal compartment, inasmuch as such an expression of HO-1 is evident as well in macrophages in red pulp of spleen, another major compartment for the erythrocyte removal and heme degradation [2]. On the other hand, the liver parenchyma is considered to be a major cellular compartment for localization of non-hemoglobin heme proteins such as cytochrome P450 [21]. Since heme molecules of these enzymes are known to be metabolized almost exclusively by the heme oxygenase reaction [2], it is not unreasonable to suggest that, in the normal liver, HO-2 limits intrahepatic turnover of the heme enzymes.

Considering a compartmentalization of CO generation characterized by intra- and extra-sinusoidal distribution of specific HO isoforms, these lines of knowledge on heme degradation could provide a clue to refine the ideal design of blood substitutes. Intra- or intermolecular crosslinking is thought to consider a potential strategy to prevent renal ultrafiltration end to elongate the halflife of blood substitutes. In organs possessing fenestrated endothelial cells such as the liver, however, sieve pores could allow stroma-free hemoglobin molecules to access the space of Disse and scavenge the vasodilating gaseous monoxides. Furthermore, the stroma-free hemoglobins are unlikely to be catabolized in the HO system in Kupffer cells, where heme molecules derived from scenecent erythrocytes are mainly degraded, but would be degraded in the parenchymal cells. Such circumstances might jeopardize hepatocytes against oxidative stress and cause unnecessary expression of the inducible HO system and CO generation. Crosslinking of hemoglobins could delay a physiologic turnover of heme degradation by the HO system and, once administered, would stay in the extrasinusoidal space as a scavenger of the gaseous vasodilators. These possibilities should carefully be examined in the liver under normal and disease conditions such as endotoxemia or hemorrhagic shock, where HO-1 or NO synthase are overexpressed prior to the administration of the Hb derivatives. However, the present study suggest that liposomal encapsulation of the Hb derivatives serves as a potential strategy to reduce these risks to perturb the hepatic microvascular function.

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CHAPTER 24

Oxygen-Transport Albumin: A New Hemoprotein Incorporating Lipidhemes as a Red Cell Substitute

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Introduction

In mammals, serum albumin is synthesized in the liver and possesses a half-life in circulation of ca. 19 d. As the most abundant protein in the circulating system and with a typical concentration of $5\,g\,dl^{-1}$, it contributes 80% to the colloid osmotic pressure. The most outstanding property of serum albumin, on the other hand, is to transport an incredible variety of endogenous and exogenous compounds. Serum albumin furthermore performs many other functions as well, such as maintaining the blood pH, etc. Numerous reviews have already dealt with albumin's structure, functions and metabolism [1–5].

The albumin molecule is somewhat like a sponge. Among many substances which bind with a high affinity to albumin, the biologically important ligands are especially long-chain fatty acids and bilirubin. The interaction results in an increased solubility in plasma of both compounds. The toxicity of bilirubin is therefore decreased in this way.

Hemin released from hemoglobin is also transported to the liver by serum albumin for metabolic processing. The binding equilibria of porphyrin derivatives to albumin have been studied over the last two decades [6–11]. It has been clarified that serum albumin contains one binding site of hemin with high affinity, as well as additional sites with much lower affinity. The static spectrum of albumin-protoheme is intermediate between that of free heme and that of hemoglobin. Casella et al. clarified that the binding of the hemin to HSA involves coordination of a histidine residue of the protein to one of the iron axial coordination sites [12].

One may expect some novel functions of these albumin-heme complexes; however, little interest has been generated so far [6]. The hemopexin which is present in plasma binds free heme in both iron(II) and iron(III) forms, and the iron(II) complex definitely binds oxygen and CO reversibly, for example. Marden et al. demonstrated that the reduced deoxy human serum albumin (HSA)-protoheme complex formed a stable CO adduct and showed a typical geminate rebinding reaction after laser-flash

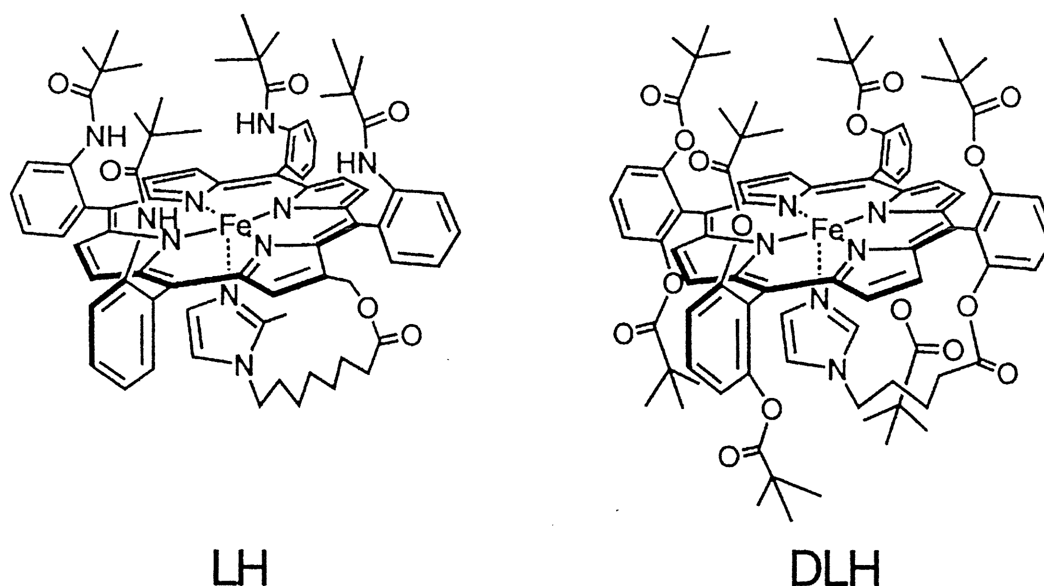

Fig. 1. Structure of lipidhemes.

photolysis [13]. Nevertheless, the oxygenated species could not be detected. Bonaventura et al. preliminarily reported the reversible spectral change of HSA with tetrakis(*o*-pivalamido)phenylporphinatoiron(II) in the presence of an excess of axial imidazole upon exposure to oxygen [14]. Their structure and the O_2-binding formation have, however, not been clarified.

We have recently found that a tetraphenylporphinatoiron(II) derivative with an intramolecular coordinated axial base, 2-[8-{*N*-(2-methylimidazolyl)}octanoyloxymethyl}-5,10,15,20-tetrakis($\alpha,\alpha,\alpha,\alpha$-*o*-pivalamido)phenylporphinatoiron(II) (lipidheme: LH, Fig. 1) [15] is efficiently incorporated into the HSA, providing a synthetic hemoprotein, which can bind and release oxygen reversibly under physiological conditions (in aqueous media, pH 7.4, 37°C) like hemoglobin [16–18]. In this chapter, the performance of this new type of O_2-carrying molecule, HSA-LH complex, as a red cell substitute are described.

Structure and ligand binding aspects of serum albumin

After numerous efforts have been made to solve the three-dimensional structure of HSA, Carter et al. reported a new tetragonal crystal that eventually proved suitable for structure determination in 1989 [19]. Furthermore, HSA was among the very first proteins crystallized in the microgravity environment in space [20]. To date, there have been several albumin structures determined by crystallographic methods. In contrast to the early conception of an oblate ellipsoid shape (140 × 40 Å), the crystal structure of HSA reveals a heart-shaped molecule which can be approximated to an equilateral triangle with sides of 80 Å and a depth of 30 Å (Fig. 2) [21]. It has been

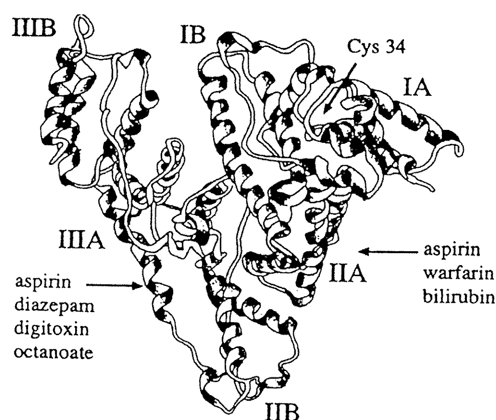

Fig. 2. Stereo view of HSA molecule (ref. [21]).

elucidated that HSA has 3 homologue domains (I, II and III) with 9 loops formed by 17 disulfide linkages, and each domain is constructed of 2 subdomains (IA, IB, etc.). The details of the HSA structure are referred to in Carter's reviews [22].

Based on an interest in the non-specific molecular binding aspect, albumin research involving binding studies has been widely developed e.g. [1]. According to the general consensus, there are six dominant regions of ligand association with albumin. The majority of the ligands are bound in one or both sites within specialized cavities of subdomains IIA and IIIA [22]. Most ligands are bound reversibly with typical equilibrium constants (K) ranging from 10^6 to 10^4 M^{-1}.

Albumin is recognized as the principal transport protein for fatty acids and other lipids that would otherwise be insoluble in the plasma. The binding constants are well-known for a variety of saturated and unsaturated fatty acids. The total fatty acid capacity of albumin varies with its chain length but averages ca six per albumin molecule. Under normal physiological conditions, one or two fatty acids are absorbed by albumin [4]. There are, however, no clear conclusions about the exact location of the bound long-chain fatty acids. A crystallographic study of fatty acid binding to HSA is now being undertaken by Carter et al.

On the other hand, it is almost accepted that bilirubin is primarily bound to a site within IIA. Concerning hemin, Hrkal et al. reported that the primary binding site is located in the sequence 124–298, which corresponds to subdomains Ib, IIa [23].

Human serum albumin-lipidheme complex as a new O_2-carrying molecule

Lipidheme binding to HSA

Synthetic tetraphenylporphinatoiron(II) derivatives have been extensively used for the research on hemoprotein models, because of their stability and advantage of covalent modification [24]. For the design of hemoglobin analogs, the proximal base bound to the central iron ion, namely the imidazole, plays a crucial role in the preparation of a stable iron-O_2 complex. From this viewpoint, a series of

superstructured porphinatoiron(II)s with an intramolecular coordinated axial base, the so-called "lipidhemes" (LHs), have been continuously synthesized by the authors [15,25]. We have recently found that LH (Fig. 1) is efficiently incorporated into serum albumin, providing a synthetic O_2-carrier, which can transport oxygen under physiological conditions (pH 7.4, 37°C) as well as in vivo [16,17].

Our first interest in this new albumin-heme molecule was its nano-structure. How many and at which place in the HSA LH molecules are bound? The HSA-LH(CO) solutions were, for example, simply prepared by mixing an ethanol solution of carbonyl-LH with an aqueous solution of HSA, followed by ultrafiltration and dialysis processes [17]. On the basis of the quantitative analysis of the free carbonyl-LH molecule in the solution prepared with different LH/HSA mixing ratios (1–14), the equilibrium constants were then calculated [26,27].

The incorporation ratios were determined to be 100% for LH/HSA: 1, 99% for 4, 94% for 8, but only 60% for 14 (Fig. 3). We concluded that the maximal binding ratio of LH to one HSA molecule was *eight*. The concentration of HSA was always constant (5 g/dl) independent of the mixing ratio. The magnitude of the binding constants for the LH association with HSA (K_1–K_8) ranged from 1.2×10^6 to 1.3×10^4 M^{-1}. These values are relatively low compared to those of palmitic acid (K_1–K_8: 6.2×10^7–3.8×10^5 M^{-1}) and hemin (K_1: 5.0×10^7 M^{-1}), which afford special interactions with albumin through their carboxylic groups. The LH molecule, on the other hand, binds the albumin only by hydrophobic interaction.

The binding sites of LH were then estimated from the binding inhibition by other ligands which occupied the major association sites of HSA. Palmitic acid,

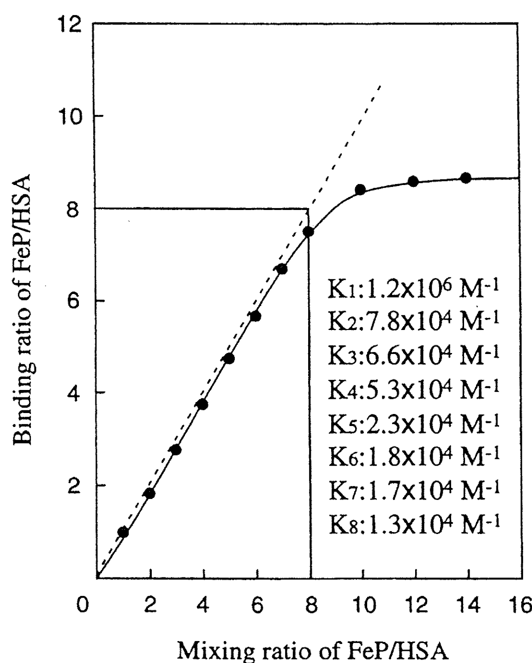

Fig. 3. Binding ratio of LH molecules to HSA.

protoporphyrin IX sodium salts instead of hemin, and phenol red instead of bilirubin were used as the inhibitors. The quantitative analysis of LH was again performed using HSA with each ligand (molar ratio: 1/1).

At an LH/HSA mixing ratio of eight, the binding numbers were significantly decreased to seven in all cases with inhibitor molecules; one of the binding regions of LH is obviously occupied by the inhibitor ligand. Consequently, the primary association sites of these ligands are identical to one of the eight binding sites of LH. It is remarkable that the LH binding was inhibited even by phenol red with a low K_1 of $2.5 \times 10^4\ M^{-1}$. This indicated that a minor binding site, at least a fifth one, of LH was definitely blocked by phenol red molecule.

Physicochemical property

The obtained red-colored dispersion could be stored at 4°C for three months and could be kept as a freeze-dried powder for more than six months. The aqueous redispersion of the powder does not show any differences in the turbidity, the incorporation ratio of LH, and the filter permeability.

The solution properties of HSA-LH are summarized in Table 1. Specific gravity varied from 1.013 (LH/HSA: 1) to 1.021 (LH/HSA: 8). The viscosity was the same as that of HSA (1.3 cP at a high share rate of $230\ s^{-1}$), which was much lower than that of human blood (4.4–5.0 cP). No aggregation was observed, even after eight LH molecules bound to one HSA.

Circular dichroism (CD) studies have provided information bearing on the secondary and tertiary structures of HSA and conformation changes that ensure when LH is bound. The spectral pattern showed typical double-minimum negative peaks in the ultraviolet region independent of the binding numbers of the LH molecules from one to eight [18]. Remarkably, there are no differences between the HSA-LH hybrid and normal HSA, suggesting that LH association does not cause any conformational changes in the albumin. The calculated α-helix content was approximately 51%, which is also the same as that of HSA itself. These observations clearly indicate that the structure of the albumin host is not affected by incorporation of LH molecules.

The binding of hemin to the specific center of the albumin molecule is generally accompanied by the rise to an extrinsic negative Cotton effect in the Soret band

Table 1

Solution property of HSA-LH solution at 37°C

System	Specific gravity	Viscosity (cP at $230\ s^{-1}$)
HSA-LH	1.013–1.021	1.1
Serum	1.027	1.1
Human blood	1.055–1.063	4.4–5.0

region, because it binds to the albumin through a covalent bond between a histidine imidazole and the iron center, allowing a high degree of immobilization [7,12]. HSA-LH did not, however, show any CD bands at the Soret region.

The isoelectric point of HSA-LH was determined to be 4.8 from the isoelectric focusing measurement, which agreed with that of HSA. The native page also showed completely the same molecular size as HSA without LH. We concluded that the structure and surface charge distribution of HSA was not changed upon binding of the LH molecules.

O_2-binding ability

The visible absorption spectral pattern of a deoxygenated HSA-LH showed a typical five-coordinated tetraphenylporphinatoiron(II) species (λ_{max}: 439, 542, 563, 605 nm), indicating that the covalently attached imidazolylalkyl group was intramolecularly coordinated to the central iron (Fig. 4). This spectrum changed to that of the oxygenated type (λ_{max}: 423, 548 nm) upon the exposure to the air or oxygen. This O_2-association and -dissociation are reversible, and the degree of oxygenation corresponds to the O_2-partial pressure; the O_2-binding affinity [$p_{1/2}(O_2)$] was 32 mmHg at 37°C. Interestingly, the $p_{1/2}(O_2)$ values are constant independent of the numbers of binding LH molecules. The carbonyl state (λ_{max}: 424, 540 nm) was also immediately generated upon exposure to carbon monoxide gas with either the deoxy or oxy state.

The autooxidation reaction of the oxy HSA-LH (λ_{max}: 548 nm), however, took place slowly and the absorption band of 548 nm almost disappeared after 24 h, leading to formation of inactive hemin. The half-life ($\tau_{1/2}$) of the oxygenated species was 7 h at 25°C and 1 h at 37°C, respectively (under air). The activation energy of the oxidation of the HSA-LH(O_2) was calculated to be 89 kJ mol^{-1}. The $\tau_{1/2}$ was increased by raising the O_2-partial pressure (e.g. 2.3 h at 37°C in a pure oxygen atmosphere). This is the same profile as that of hemoglobin [28].

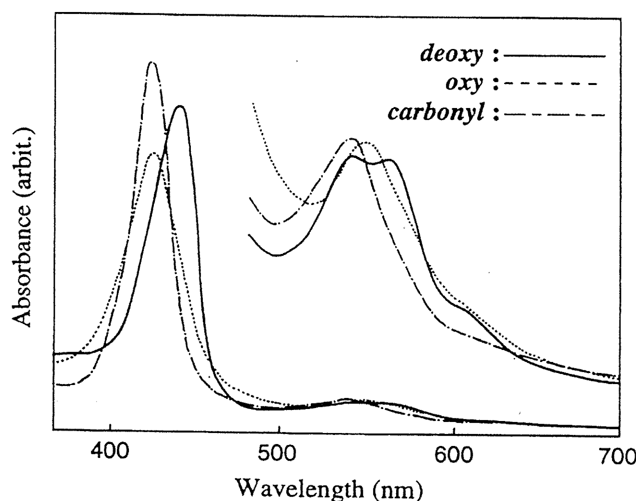

Fig. 4. Visible absorption spectral changes of HSA-LH solution.