ANTIOXIDANTS & REDOX SIGNALING
Volume 10, Number 7, 2008

DOI: 10.1089/ars.2007.1982

Editorial

How to Evaluate Blood Substitutes for Endothelial Cell Toxicity

CAROLINE GAUCHER and PATRICK MENU

ABSTRACT

The most common and widely transplanted tissue worldwide is blood. But concerns about safety and adequacy of blood transfusion have fostered 20 years of research into blood substitutes such as oxygen carriers based on modified hemoglobin (Hb). Chemically modified or genetically engineered Hb developed as oxygen therapeutics are designed to restore blood volume and to correct oxygen deficit due to ischemia in a variety of clinical settings. Uncontrolled oxidative reactions mediated by large amounts of cell-free Hb and their reactions with various oxidant/antioxidant and cell signalling systems emerge as an important pathway of toxicity. Hemoglobin can react with oxygen and NO, leading to the production of reactive oxygen or nitrogen species. Inside the bloodstream, oxidized Hb and ROS/RNS are in direct contact with endothelial cells (EC). Thus, chain reactions may trigger molecular and cellular biology, causing oxidative stress-related pathologies. This editorial presents an overview of interactions between Hb (modified or not) and EC. We also propose a wide range of techniques and methods to assess oxidative stress and inflammation responses of EC after exposure to Hb. This editorial can serve as a guide to evaluate *in vitro* toxicity of new Hb molecules. *Antioxid. Redox Signal.* 10, 1153–1162.

SAFE BLOOD TRANSFUSION, A CHALLENGE FOR THE FUTURE

BLOOD TRANSFUSION IS ASSOCIATED with significant adverse effects such as potential virus transmission. Furthermore, as the need for fresh blood for transfusion increases, the requirement for an easily produced artificial blood substitute becomes evident. After several decades of intensive efforts, research has not succeeded in producing a safe oxygen carrier.

Hemoglobin-Based Oxygen Carriers (HBOC) transport oxygen, that indicates their use when blood loss is extensive (*i.e.*, volume replacement must be associated with oxygen contribution). They are derived from purified hemoglobin (Hb), which is an obvious candidate as a blood substitute with a number of desirable characteristics. Hemoglobin is the natural protein transporting O_2 from lung to organs with a high capacity for O_2 transport and release. It lacks the numerous and complex antigens of the red blood cells (RBC) membrane, hence it appears to be universally compatible. It is a "robust" molecule, stable under standard storage conditions *(i.e.*, frozen or lyophilized).

The first clinical trial using unmodified Hb was undertaken by Amberson and colleagues in 1949 (4), and indicated complications such as severe kidney toxicity. Improved purification alleviated some, but not all the adverse effects of Hb. However, due to the purification methods used to remove endotoxins and cellular ghosts, 2,3-diphosphoglycerate (2,3-DPG) was also removed, which resulted in a decrease of O_2 delivery (23). Furthermore, free Hb when released in the circulation and diluted in the plasma can dissociate into dimers (43), resulting in a short circulatory half-life and renal toxicity. This problem has been addressed by a variety of chemical modifications, such as polymerization, cross-linking, conjugation with macromolecules like polyethylene glycol (PEG) or dextran, hybridization with

Groupe Mécanique et Ingénierie Cellulaire et Tissulaire, Faculté de Médecine, Vandoeuvre-lès-Nancy, France.

iron–cobalt, nickel–iron, or iron–zinc, and encapsulation into phospholipid vesicles (6, 40, 52, 85).

Intravenous injection of purified Hb results in chest pain, bradycardia, hypertension, decrease in urine output and endogenous creatinine clearance (75). Subsequently, Hb was chemically modified by polymerization with glutaraldehyde to increase molecular weight in order to increase vascular retention time and decrease renal toxicity. Examples of such first-generation polymerized Hb products are Polyheme® (Northfield laboratories, Inc., Evanston, IL), Hemopure® (Biopure corporation, Cambridge, MA), and Hemolink® (Hemosol Corporation, Mississauga, Canada) (93). Given the heterogeneity of the glutaraldehyde reaction and possible secondary reactions (53), research has focused on a simpler molecule, the $\alpha\alpha$-Hb (Baxter Healthcare, Deerefield, IL). This compound was stabilized by intramolecular cross-linking between α chains by bis(dibromosalicyl)fumarate (DBBF), so that neither polymerization nor subunit dissociation was possible. Unfortunately, $\alpha\alpha$-Hb was shown to be intensively vasoconstrictive (38).

A number of new and largely unresolved safety-related problems have arisen during preclinical development and in clinical trials of the current generation of HBOC. This includes vasoconstriction (27, 82), pro-inflammatory activity, and oxidative stress (35) (Fig. 1). At the time of writing, therefore, there is no blood substitute available for a "safe" human transfusion. However, the need for blood substitutes remains a current problem.

Free Hb suitable for HBOC production may be extracted from bovine or outdated human blood or produced by genetically modified microorganisms (bacteria or yeast) (Table 2). The latter technique is a relatively new and exciting avenue of research. Indeed, it allows the production and the modification of Hb without any contamination [*e.g.*, human immunodeficiency virus (HIV) or bovine spangiform encephalopathy (BSE)] or, because cross-linking may be achieved by genetic manipulation, chemical products. A European project entitled "Euro Blood Substitutes" (EBS) (www.eurobloodsubstitutes. com, accessed 2/15/08) was funded within the European Union 6th Framework Programme. The EBS Project aims to develop a technological platform for producing novel heme proteins and blood substitute components using micro-organisms as "cell factories."

When injected intravenously, HBOC come into contact with blood cells but also with the endothelium, which covers the luminal surface of blood vessels from the heart to the smallest capillaries. The endothelium is a biologically active monolayer of endothelial cells (EC), providing an interface between blood flow and tissues. They are involved in vasomotion through synthesis of nitric oxide (NO), for example, homeostasis through interaction with platelets, atherosclerosis, angiogenesis, and inflammation via the expression of membrane receptors for leukocytes. EC can sense the physical and/or chemical properties of blood substitutes and regulate their behavior.

Publications dealing with HBOC/EC interactions refer to EC from various origins (bovine or human), extracted from diverse

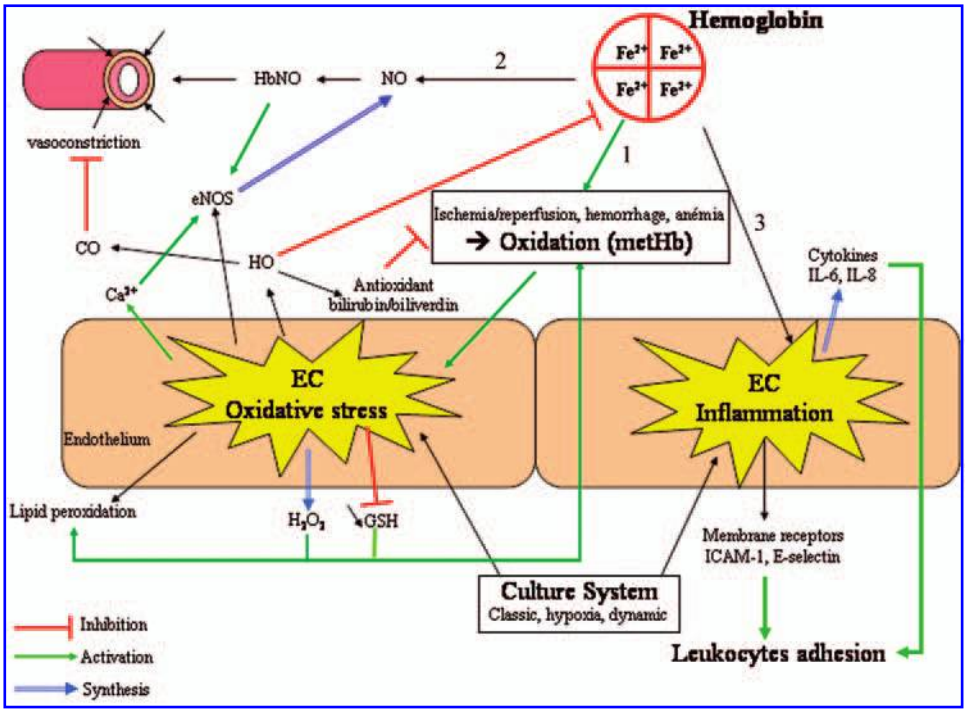

FIG. 1. Oxidative stress, inflammation, and adaptation of endothelial cells. **1.** Hemoglobin (Hb) is oxidized into methemoglobin (metHb) by oxidative stress engendered by hemorrhage, ischemia/reperfusion, or anemia. Oxidative stress on endothelial cells (EC) induces lipid peroxidation, H_2O_2, and Ca^{2+} leakage from the cells. It lowers reduced glutathione (GSH) concentration. MetHb upregulates the transcription and activity of the heme-oxygenase-1 (HO-1). This leads to reduce the oxidative stress (biliverdin) and vasoconstriction (CO). **2.** Hb can scavenge NO, decreasing its bioavailability and inducing vasoconstriction. **3.** Hb can induce inflammatory reactions and potentiate leukocytes recruitment by EC.

Table 1. Different Types of Endothelial Cells Used in the Studies Quoted in Text

Type	*Signification*	*References*
BAEC	Bovine aortic EC	20–22, 44, 71
BPAEC	Bovine pulmonary artery EC	117
HAEC	Human aortic EC	102
HCAEC	Human coronary artery EC	98
HMVEC	Human lung microvascular EC	18
HUVEC	Human umbilical vein EC	34, 97, 108, 109

EC, endothelial cells; N.D., non determined.

tissues (arteries or veins) (Table 1). cause structure and phenotypes of EC vary between animals and segments of the vascular tree (3), EC origin makes a huge difference in experimental results. One can wonder what is the interest to use animal cells in the place of human cells. The greater choice should be between the different sites of the vasculature. Arteries, veins, and microvessels are not submitted to the same oxygen partial pressure and do not have the same structure. Indeed, the main functions of the endothelium are differentially regulated between different sites of the vascular tree and from one moment to the next (2). For example, endothelial nitric oxide synthase expression is higher on the arterial side of the circulation of organs (72). The choice of EC type should be driven by the application of the HBOC tested.

Experimental procedures leading to the FDA approval of a new pharmacological molecule start from (a) physico-chemical characterization of the product, (b) *in vitro* activation of cells, (c) *ex vivo* experiments on isolated organs, (d) *in vivo* tests, and end with clinical evaluation in humans (35). Paradoxically, few studies on the *in vitro* impact of blood substitutes on EC are available in the literature.

One of our purposes is to assemble or facilitate access to a substantial part of the information a scientist needs to monitor HBOC interactions with EC *in vitro*. We hope to be as extensive as possible on all phenomena observed. This editorial completes a recent review published on this subject (10). Our reflections are a guide for *in vitro* evaluation of the new Hb toxicity.

OXIDATIVE STRESS, THE JANUS FACE OF HEMOGLOBIN

Hb: a versatile protein

Aerobic organisms are endowed with enzymatic oxidant defense systems, which provide protection against activated oxygen species. Damage caused by reactive oxygen species can be greatly amplified if free redox active iron is present (37, 100). One abundant source of potentially toxic iron is exogenous (HBOC) and endogenous heme, which can enhance oxidant-mediated cellular damage (7, 8, 36, 66, 73).

HBOC are used to reoxygenate organs and tissues when the blood loss is so extensive that it perturbs hemodynamic parameters. Indeed, blood oxygen carrying capacity is so low that the metabolism becomes anaerobic, inducing oxidative stress. HBOC injections complicate the situation because they typically have a higher affinity for O_2 than Hb inside RBC. So, the amount of oxyhemoglobin (HbO_2) in the blood vessels is higher and, because oxidative stress is already engaged and HbO_2 has the capacity to auto-oxidize, the production of reactive oxygen species is substantial. Moreover, the injection of free Hb into the bloodstream can overload the Hb detoxification system.

Hydrogen peroxide (H_2O_2), produced by superoxide dismutase, is reportedly able to generate hydroxyl radicals when it reacts with free ferrous iron (Fe^{2+}) oxidizing to ferric Fe^{3+} (Fenton reaction). H_2O_2 can also convert oxyhemoglobin (Fe^{2+}) into methemoglobin (Fe^{3+}), which cannot transport O_2 and is toxic (32). H_2O_2 can also react with metHb as well as other heme proteins to produce a higher oxidation state of the iron, ferryl or Fe^{4+} (20, 31, 33, 42), which can initiate membrane lipid peroxidation and/or oxidize other macromolecules.

Oxidative stress plays a major role in the pathogenesis of EC, inducing dysfunction such as hypertension, inflammation, and atherosclerotic cardiovascular diseases. Because of their direct and continuous contact with the circulating blood, EC are particularly sensitive targets for oxidative products generated by free Hb. Several *in vitro* studies showed that Hb (at low concentration) either can be directly cytotoxic or can alter the EC biology inducing cytoskeleton alterations (15) and sensitization to H_2O_2 (9).

Purified Hb or HBOC preparations in their reduced forms (Fe^{2+}) are not, by themselves, cytotoxic to EC (17, 18). However, the highly reactive Fe^{4+} and, to a lesser extent, its Fe^{3+} forms can induce significant cytotoxicity. EC contain a protective system to detoxify the blood from Hb leakage out of the RBC (48). Part of this system includes heme oxygenase.

Endothelial cell self-defense: heme oxygenase

Heme oxygenase (HO) was discovered in the 1960s (80) as the major heme catabolic enzyme that converts heme into biliverdin and carbon monoxide (CO), and releases iron. Although HO contributes to homeostasis of heme metabolism by oxidizing any excess of heme released from senescent RBC, it also appears to play an important role in defense against oxidative stress. Indeed, HO produces biliverdin and bilirubin (the reduced form of biliverdin), which are two physiologically effective antioxidants.

Three HO isozymes HO-1, HO-2, and HO-3 have been described (54, 76). HO-1, which is distributed ubiquitously in mammalian tissues, is induced strongly and rapidly by many compounds that elicit cell injury like inflammatory cytokines, oxidants, and NO (5, 22, 45). Furthermore, HO-1 induction is an indispensable *in vivo* response in the protection of kidneys against acute heme protein toxicity (62). The natural substrate of HO, heme, is itself a potent inducer of the enzyme (96). Furthermore, ferric heme is known to increase HO-1 mRNA level (76). So, HBOC auto-oxidation into metHb can also induce HO-1.

Cheng and collaborators demonstrated that polymerized Hb (Polyheme, Northfield Laboratories) induces the synthesis of HO-1 protein in a time-dependent manner in human lung microvascular endothelial cells (HMVEC) (15). Wagener and collaborators found that the contribution of HO-1 and HO-2 isoforms to the total HO activity in human umbilical vein

endothelial cells (HUVEC) exposed to heme was 70% due to HO-1 and 21% to HO-2 (89).

HO-1 also increases the production of endogenous CO, inducing vasodilation (90, 68). Hb is known to scavenge NO, thus decreasing its physiological availability and causing vasoconstriction. So HO-1 induction by modified Hb, by enhancing the production of CO, can reduce the effect of NO scavenging by heme and diminish the consequent vasoconstriction.

The delicate balance of nitric oxide

NO was discovered to be a potent vasodilator in 1979 (34) and later identified as the endothelium-derived relaxing factor (EDRF) (41, 67). It is synthesized *in vivo* from L-arginine by three NO synthases isoenzymes (NOS), including neuronal (nNOS, NOS I), inducible NOS (iNOS, NOS II), and endothelial NOS (eNOS, NOS III). A mitochondrially-localized NOS (mtNOS) was discovered in 1995 (47) and recently identified in EC (21). It produces more NO than eNOS do (13). For the moment, no publication on NO produced by mtNOS due to the presence of HBOC is available.

eNOS is expressed in EC as a constitutive enzyme and produces picomolar to nanomolar amounts of NO for short periods in response to receptor stimulation (acetylcholine, bradykinin) or shear stress. NO through its vasodilator activity is crucial for the maintenance of vascular homeostasis. Moreover, it has recently been discovered that RBC exhibit an active and functional endothelial type NO synthase. RBC-NOS regulates deformability of the RBC membrane and inhibit activation of platelets (46). NO is a puissant molecule interacting with blood cells (RBC, platelets, leukocytes), and vascular wall (endothelium, smooth muscle cells) (91). While excessive NO production is generally associated with tissue injury, it is important to note that NO constitutively produced by endothelium is believed to play a protective role in the microvasculature by inhibition of platelet and neutrophil adhesion to endothelial monolayers (1).

Preclinical and clinical studies reported that the major adverse effect induced by Hb solutions is a transient vasoconstriction (87), presumably due to NO scavenging by heme. Another situation that decreases NO concentration in the bloodstream is its reaction with reactive oxygen species which promotes the formation of NO_2^- (nitrite) and NO_3^- (nitrate).

The reactions between Hb and NO are important factors determining how NO works *in vivo* and may mediate the biological effects of free Hb on EC. Injection of a HBOC solution increases the amount of free Hb in the blood and promotes the reaction between NO and Hb. NO can react with either deoxy or oxyHb, forming nitrosylhemoglobin (HbNO) and methemoglobin plus NO_3^-, respectively (70).

Unmodified oxyHb induced a higher NO_2^-/NO_3^- formation ratio in normal human coronary artery EC, whereas an improved

Table 2. Different Types of Hemoglobin and Modified HemoglobinU in Studies Quoted in Text

Designation	*Origin*	*Molecular weight (kDa)*	*Modification*	*References*
Hemin	ND	ND	None	85, 93
Hemoglobin, Hb, ferrous, Oxy Hb, free Hb, stroma free Hb, HbAg, unmodified Hb	Bovine Human	64	None	13, 16, 33, 53, 54, 75, 76
Methomoglobin, MetHb, Ferric Hb, hemin	ND	64	None	7, 48, 74, 75, 85, 86
Mesoporphyrin	ND		None	74, 75
Dex-BTC-Hb	Human	200	Conjugated with dextran benzene-tetracarboxylate	11, 25, 79
$\alpha\alpha$-Hb, αDBBF-Hb, DBBF-Hb	Human	64	Intramolecularly cross-linked with dibromosalicylfimarate	17, 18, 33, 54, 62, 79
Polymerized α-DBBF-Hb	Human	400	Polymerization with bis(maleoglycylamide) polyethylene (BMAA-PEG)	33
Polynitroxylated $\alpha\alpha$-Hb	Human	ND	Polynitroxylated	62
Liposome encapsulated Hb	Human	212 nm	Encapsulation	59, 95
Hb polytaur	α-Human and β-bovine		Polymerization of Hb-minotaur	23
PolyHb, polyheme	Human	ND	Polymerization	14
o-Raffinose cross-linked Hb, hemolink	Human	128	Cross-linked with o-raffinose	94
Hb cross-linked intra- and intermolecularly with o-adenosine	Bovine	ND	Crosslinked intra- and intermolecularly with o-adenosine	76
Erythrocytes lysates	Human	ND	Lysis	15, 94

blood substitute consisting of Hb crosslinked intra and intermolecularly with o-adenosine decreased it (78). So unmodified oxyHb can scavenge endothelial NO. Finally Simoni *et al*. reported that Hb induced hemodynamic changes mediated, in part, by the disruption of NO vasodilator activity (78).

eNOS transcription and translation could balance NO scavenging by HBOC infusions. HAEC incubated for 3 h in the presence of two different Hb derivatives, Dex-BTC-Hb and $\alpha\alpha$-Hb, did not exhibit any change of mRNA or protein expression (81).

To show the impact of different substances on vasomotion, experimental models of veins or arteries have been developed consisting of vascular rings mounted in tissue baths. Exposure of vascular rings to Hb results in vasoconstriction (33, 60, 86). Chavez–Negrete and co-workers showed that there was no difference between stroma-free Hb and starch-hemoglobin (MW: 200–260 kDa) on the *in vitro* contractile activity of adult male rats aortic rings (14). Their findings do not support the hypothesis that an increase of Hb molecule size prevents Hb extravasation, and the consequent vasoconstriction due to NO scavenging by Hb in the cellular space between endothelium and smooth muscle cells.

iNOS was originally discovered in macrophages (94) but is also expressed in EC. It is induced by proinflammatory cytokines (29, 83) and shear stress (65). It generates significantly greater and more sustained amounts of NO compared to eNOS. The micromolar concentrations generated by high-output iNOS are microbicidal as well as pro-inflammatory, damaging to the surrounding cells and tissues. iNOS is thought to contribute to the S-nitrosylation of Hb (63).

A fight for survival

Hb is a heme protein exhibiting catalase and peroxidase activities (30, 69). Cytotoxic activity is displayed by all peroxidases tested so far, as well as by various degradation products of heme proteins, including microperoxidase and hemin (7). The enzymatic system responsible for cell death consists of the cytotoxic triad: myeloperoxidase, H_2O_2 generated by the NADH oxidase, and endogenous chloride (halide ion) (25). It is now generally accepted that this mechanism involves an oxidation of the halide to the corresponding hypohalous acid by the peroxidase (57). The hypohalous acid (HOCl, HOI) then attacks cells in a series of nonenzymatic reactions, leading to its destruction.

The peroxidase activity of hemoproteins involving the ferryl heme intermediate is well recognized (Hb cycles between Fe^{3+} and Fe^{4+} as it consumes H_2O_2); this activity has been detected in human and animal whole blood by electron paramagnetic resonance microscopy (79).

Oxidized Hb cytotoxicity varies from cells injury to cell death. EC incubated with oxidized Hb become round, showing a detachment of the cells from the culture surface and cell death (19). In a model of hypoxia reoxygenation with α–DBBF Hb, McLeod *et al*. showed that cell survival depends on the ischemia period and on the Hb concentration used during reoxygenation (56). α–DBBF Hb at 60 and 100 μM did not affect cell survival, whereas 200 μM increased toxicity on both control and cells subjected to varying periods of hypoxia–reoxygenation.

Further molecular mechanisms concern DNA fragmentation, cell cycle arrest, and alteration of DNA content. Goldman and collaborators demonstrated that DNA fragmentation occurring in EC after a contact with H_2O_2 can be decreased by addition of free hemoglobin (HbA_0) (33). D'Agnillo and co-workers showed that HbA_0 and DBBF-Hb reduce the number of early apoptotic cells treated with H_2O_2 (17). The effect of DBBF-Hb is lower than HbA_0 one. This indicates that there are two levels of Hb implication: (a) Hb catalytically lower tissue H_2O_2 and alter the course of death, (b) Hb can be directly toxic to cells, oxidizing into more reactive oxidation states (ferrylHb). Furthermore, they concluded that the apparent difference in the pro-apoptotic effects between the two Hb may reflect their different rates of H_2O_2 consumption. D'Agnillo and collaborators investigated the cytotoxicity of DBBF-Hb and glucose oxidase-generated H_2O_2 (19). They showed that the redox cycling of DBBF-Hb by low levels of enzymatically generated H_2O_2 induces G2/M cell cycle arrest, which was followed by apoptosis of growth-arrested cells.

Impairment of cells integrity/metabolism

Glutathione (GSH) concentration remains a great indicator of oxidative stress. Unmodified Hb seems to decrease intracellular content of GSH (78). This suggested that it creates an oxidative stress and free radical production, which oxidized GSH into GSSG. Moreover, the combination of H_2O_2 with HbA_0 or DBBF-Hb produces a significant decrease in GSH contents compared to H_2O_2 alone (17). D'Agnillo and co-workers concluded that the formation of ferryl intermediate was closely linked to a greater loss of GSH (17). The enhanced loss of GSH may be explained by the aggravated peroxidative stress induced by heme-mediated lipid peroxidation processes, or possibly the efflux of GSH from stressed cells. Furthermore, McLeod and co-workers proved that reoxygenation during 3 h with free Hb caused depletion of intracellular GSH in a concentration-dependent manner (56).

EC produce H_2O_2 under normal conditions and to a greater extent after anoxia or ischemia followed by reperfusion (100). Oxidation of Hb by H_2O_2 has been correlated with oxidative stress and cell injury (58). H_2O_2 converts carboxylic acids into peroxy acids, which are used as oxidizing agents (44). This phenomenon is part of lipid peroxidation.

Unmodified Hb increases the production of lipid hydroperoxides (LOOH) in human coronary artery endothelial cells (HCAEC), but Hb crosslinked intra and intermolecularly with o-adenosine is nontoxic (78). McLeod and collaborators demonstrated the correlation between the ischemic period on BAEC and the quantity of lipids peroxidized (56). They also showed that addition of α–DBBF Hb caused a significant increase in lipid peroxidation after 2 h of hypoxia, followed by 1 h of reoxygenation.

Lactate is an indicator of cells suffering during hypoxia/reoxygenation. McLeod and collaborators (56) showed that lactate production was increased in BAEC during hypoxia, and dependent on the duration of hypoxia. Simoni and co-workers compared the cytotoxic effects of different Hb oxidation states (ferrous, ferric, ferryl) and molecular weights (400–1,020 kDa) on HUVEC (77). The lactate dehydrogenase level was highest following incubation with ferrylHb and lowest with low mo-

lecular weight polymerized Hb. So, the oxidation state of iron in Hb plays an important role in EC injury induction.

The intracellular signal that links systemic or local external stimuli to the synthesis and release of vasoactive compounds such as endothelins and EDRF, appears to be the level of Ca^{2+} which activates Ca^{2+}-dependent enzymes like eNOS. High levels of $[Ca^{2+}]i$ are toxic and may affect cell function and cause cell death.

Simoni and co-workers demonstrated that unmodified Hb significantly increases $[Ca^{2+}]i$ in HUVEC in comparison with purified Hb crosslinked intramolecularly with o-adenosine triphosphate and intermolecularly with o-adenosine. They concluded that an increase in $[Ca^{2+}]i$ may be triggered by Hb mediated generation of oxygen free radicals (77). Zhang *et al*. tested Hemolink as a purified Hb *versus* erythrocyte lysate on BPAEC (97). Hemolink produced a very weak elevation in $[Ca^{2+}]i$ compared to erythrocyte lysate. Erythrocyte lysate is known to be cytotoxic (16) so they suggested that this toxicity can be related to reactive oxygen radical generation from the lysate.

In conclusion, oxidative stress is one of the major consequences of Hb injection in human blood circulation. Indeed, Hb can be oxidized, even if modified, into met or ferryl Hb. These two compounds can provoke chain reactions such as heme oxygenase activation, or deleterious effects to EC such as morphological modification, lipid peroxidation, or cell death. Hb can also react with NO to form HbNO and become no longer physiologically available, H_2O_2 to form Hb higher oxidation states. Hb can cause an imbalance in the concentration of glutathione, lactate after hypoxia or calcium.

INFLAMMATION AS A BELL FOR RECRUITMENT

EC are a control center of the propagation of inflammatory reactions because their major role is to amplify the immune response (92). Inflammation induces the release of cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF) from various immune cells activating EC to upregulate receptors (VCAM-1, ICAM-1, and E-selectin). Receptor upregulation increases extravasation of neutrophils and monocytes to the infected site (diapedesis).

Inflammatory mediators

EC are activated by interleukins (IL-1, IL-4) and tumor necrosis factor-α (TNFα) liberated by macrophages and other white cells (11, 71). Following this activation, EC express inflammatory mediators like IL-6 and IL-8 (39, 51). Zhu and collaborators showed that IL-6 mRNA increases 30 min after the incubation of murine endothelioma cell line with encapsulated Hb (98) with a maximum at 4 h. More recently, Liu *et al*. detected an increase of IL-6 and IL-8 released by HUVEC after incubation with methemoglobin (51).

Adhesion molecules for leukocytes attraction

Membrane receptors are divided in two families: selectin and cell adhesion molecules (CAM) expressed on EC during the inflammation and implied in leukocyte diapedesis. E-selectin is the earliest mediator of leukocytes rolling on EC. CAM like ICAM-1 and VCAM-1 mediate firm adhesion between leukocytes and endothelium. The expression of adhesion molecules by EC during activation is the most studied phenomenon in the inflammation induced by HBOC.

It has been demonstrated that an oxidant can increase ICAM-1 expression. Indeed, HUVEC cultured *in vitro* with 0.1 m*M* H_2O_2 showed a two- to threefold increase in both ICAM-1 protein and mRNA between 0.5 and 2 h of stimulation (50).

Wagener *et al*. demonstrated that mesoporphyrin (a heme oxygenase inhibitor imitating Hb) increases ICAM-1 expression on HUVEC in a time and concentration dependant manner (89). The oxidation state of Hb is an important factor to predict the expression of inflammatory receptors on EC. Indeed, heme and methemoglobin are able to upregulate ICAM-1, VCAM-1, and E-selectin (49, 88) whereas polyHb decrease the inflammatory effect of TNF-α on HMVEC (15). Moreover, modified Hb such as Dex-BTC-Hb or $\alpha\alpha$Hb did not affect ICAM-1 expression (81).

Leukocyte adhesion

To our knowledge, few publications on the *in vitro* impact of HBOC on EC/leukocytes interactions are available in the literature. McFaul *et al*. showed that incubation of leukocytes with HbA_0 stimulates the release of different cytokines (IL-8, IL-6, and TNF-α) (55). Furthermore, they demonstrated that plasma from blood incubated with HbA_0 exhibited chemotactic activity and stimulated HUVEC to become adherent to neutrophils.

Okayama and co-workers highlighted the antioxidant-anti-inflammatory effect of polynitroxylated $\alpha\alpha$-Hb (PNH) (64). They compared the effects of $\alpha\alpha$-Hb and PNH on xanthine oxidase and H_2O_2-induced neutrophil-endothelial adhesion *in vitro*. Peroxide and superoxide generated by xanthine oxidase enhance endothelial/neutrophil adhesion. $\alpha\alpha$-Hb increases the release of these two products whereas PNH inhibited it.

When Hb directly meets endothelial cells

Extravasation of Hb and change of vascular permeability have been associated with the use of HBOC. A precise quantitative assessment of these phenomena, due to oncotic pressure modifying the hydrostatic equilibrium between compartments, is fundamentally important toward understanding HBOC intravascular behaviour and oxygen delivery potential.

Dull and colleagues (24) demonstrated that polymerized Hb solutions (Hb-Polytaur and Hb-(Polytaur)n) induced endothelial barrier dysfunction like complete loss of restricted diffusion across monolayers of capillary EC.

Nakai and collaborators created a double chamber separated by an EC monolayer grown on a microporous filter to measure permeability characteristics of Hb derivatives and effects of their molecular mass (from free to liposome encapsulated Hb) (61). After 60 min of incubation, it was demonstrated that Hb derivatives with small molecular mass have increased permeabilities. Intramolecular crosslinking slightly reduced the permeability coefficient compared with free Hb. The biggest molecules (PegHb and liposome encapsulated Hb) are less able to

cross the EC monolayer. This result suggests that EC are a selective barrier able to decrease the diffusion of high molecular weight HBOC, defending a better intravascular half-life of high molecular weight Hb.

Furthermore, it has been proposed that the formation of HbNO could potentiate the uptake of Hb by EC (60). However, the presence of free Hb was detected inside guinea pig aortic EC after injection of Dex-BTC-Hb (26) in an animal treated with N-nitro-L-arginine methyl ester (L-NAME), a NO synthase inhibitor (74). Faivre–Fiorina and collaborators concluded that the formation of HbNO does not enhance the uptake of Hb by EC and is not a prerequisite for Hb entry (26).

CULTURE SYSTEMS OR CONDITIONING ENDOTHELIAL CELL REACTIONS FACE TO HB

There are various techniques to cultivate EC to test blood substitutes. The classic technique consists in growing EC in a culture flask in an incubator with 5% CO_2. Several teams worked on the simulation of hypoxia/reoxygenation. They cultivated EC with 0 or 1% O_2 for different periods of time, reoxygenated with HBOC, and measured different parameters such as H_2O_2, lactate production, glutathione and lipid peroxidation (18, 56, 95).

More sophisticated systems have been developed to simulate shear stress applied on the vessel wall by bloodstream (12, 84, 99). Strangely, to our knowledge, no publication is available on the evaluation of HBOC impact on EC submitted to shear stress. Our team developed a laminar flow chamber to study mechanotransduction in EC (28, 59). We adapted this system to HBOC and are currently collecting data on the interaction between HBOC and sheared HUVEC.

THOUGHTS AND REMARKS

The most important goal for the future is to create a safe blood substitute for human transfusion without any or low secondary effects like vasoconstriction and oxidative stress. Vasoconstriction is a phenomenon thus far attributed to NO scavenging by Hb, but a new interesting explanation has been recently discovered. Indeed, vasoconstriction would be due to Hb extravasation from blood vessels and a too high P_{50} (82). This demonstrates that the fundamental mechanisms of vasoconstriction induced by free Hb are not totally understood and need to be reconsidered using fundamental models like cells and organs culture. Oxidative stress is the second main side effect of blood substitutes. Indeed, the iron present in the Hb can auto-oxidate, leading to great oxidative stress toward the endothelium.

Chemical and/or genetic modifications of Hb can suppress or enhance these reactions. For the moment, researchers are working on the expression of modified Hb into microorganisms. This is the aim of the EBS project. The project will span 3 years of intensive research into the development of a technological baseline for producing blood substitute components (novel heme proteins) using micro-organisms such as bacteria and fungi. The EBS Project will provide a technological baseline to use micro-organisms as cell factories for the production of a much needed, effective blood substitute that will help solve the problems associated with using donor blood for transfusions. Such technology has the capacity to produce a 'tailor-made' blood substitute with novel properties. For every new molecule designed, *in vitro* tests have to be performed on cells to measure potential interactions. Despite its necessity in terms of screening new molecule toxicity and impact, few publications on the measure of EC interaction with blood substitutes are available. One can wonder why such experiments are not a systematic scheme for Hb development. *In vitro* tests on EC or vessels rings and co-culture models should serve to understand fundamental mechanisms of Hb side effects (vasoconstriction, oxidative stress). We recently proposed a new system to reconcile *in vitro* and *in vivo* studies by applying Hb on EC under shear stress to mimic blood flow (28). This system enables researchers to investigate variation of blood viscosity, shear stress, and Hb concentration, and other parameters before starting investigations on more complexe models such as animals, a way for a more bioethical research.

ACKNOWLEDGMENTS

The authors thank the EuroBloodSubstitutes project for supporting this work.

ABBREVIATIONS

BAEC, bovine aortic endothelial cells; BPAEC, bovine pulmonary artery EC; $[Ca^{2+}]I$, intracellular calcium concentration; DBBF, bis(dibromosalicyl)fumarate; Dex-BTC-Hb,: dextran benzenetetracarboxylate haemoglobin; EC, endothelial cells; EDRF, endothelium-derived relaxing factor; GSH/GSSG, reduced/oxidized glutathione; H_2O_2, hydrogen peroxide; HAEC, human aortic endothelial cells; Hb/Hbs, hemoglobin/hemoglobins; HbA_0, hemoglobin A; $HbFe^{2+}/HbO_2$/OxyHb, ferrous hemoglobin/oxygenated haemoglobin; $HbFe^{3+}$/metHb, met haemoglobin; HBOC, hemoglobin based oxygen carriers; HCAEC, human coronary artery endothelial cells; HIV, human immunodeficiency virus; HMVEC, human lung microvascular endothelial cells; HO/HO-1, HO-2, HO-3, heme oxygenase/ heme oxygenase-1, -2, -3; HUVEC, human umbilical vein endothelial cells; ICAM-, intercellular adhesion molecule; IL-1, IL4, IL6, IL8, interleukin-1, -4, -6, -8; L-NAME, N-nitro-L-arginine methyl ester; LOOH, lipid hydroperoxides; MW, molecular weight; NADH, reduced nicotinamide adenine dinucleotide; Na_2S, sodium sulfide; $NO/NO_2/NO^{2-}/NO^{3-}$, nitric oxide/nitrogen dioxide/nitrite/nitrate; NOS/eNOS, NOS III/iNOS, NOS II/nNOS, NOS I, NO synthase/ endothelial NOS/ inducible NOS/neuronal NOS; $O^{2\cdot-}$, superoxide; $ONOO^-$, peroxinitrite; PEG, polyethylene glycol; PFC, perfluorocarbon; PNH, polynitroxylated $\alpha\alpha$-Hb; RBC, red blood cells; TNF, TNF-α: tumor necrosis factor; VCAM-1, vascular cell adhesion molecule.

REFERENCES

1. Abramson SB, Amin AR, Clancy RM, and Attur M. The role of nitric oxide in tissue destruction. *Best Pract Res Clin Rheumatol* 15: 831–845, 2001.
2. Aird WC. Endothelial cell dynamics and complexity theory. *Crit Care Med* 30: S180–185, 2002.
3. Aird WC. Endothelial cell heterogeneity. *Crit Care Med* 31: S221-S230, 2003.
4. Amberson W, Jennings J, and Martin Rhode C. Clinical experience with hemoglobin-saline solutions. *J Appl Physiol* 1: 469–489, 1949.
5. Applegate LA, Luscher P, and Tyrrell RM. Induction of heme oxygenase: a general response to oxidant stress in cultured mammalian cells. *Cancer Res* 51: 974–978, 1991.
6. Arifin DR and Palmer AF. Determination of size distribution and encapsulation efficiency of liposome-encapsulated hemoglobin blood substitutes using asymmetric flow field-flow fractionation coupled with multi-angle static light scattering. *Biotechnol Prog* 19: 1798–1811, 2003.
7. Balla G, Jacob HS, Eaton JW, Belcher JD, and Vercellotti GM. Hemin: a possible physiological mediator of low density lipoprotein oxidation and endothelial injury. *Arterioscler Thromb* 11: 1700–1711, 1991.
8. Balla G, Vercellotti GM, Muller–Eberhard U, Eaton J, and Jacob HS. Exposure of endothelial cells to free heme potentiates damage mediated by granulocytes and toxic oxygen species. *Lab Invest* 64: 648–655, 1991.
9. Balla J, Jacob HS. Balla F, Nath K, Eaton JW, and Vercellotti GM. Endothelial-cell heme uptake from heme proteins: induction of sensitization and desensitization to oxidant damage. *Proc Natl Acad USA* 90: 9258–9289, 1993.
10. Balla J, Vercellotti GM, Jeney V, Yachie A, Varga Z, Jacob HS, Eaton JW, and Balla G. Heme, heme oxygenase, and ferritin: how the vascular endothelium survives (and dies) in an iron-rich environment. *Antioxid Redox Signal* 9: 2119–2138, 2007.
11. Bevilacqua MP, Pober JS, Wheeler ME, Cotran RS, and Gimbrone MA Jr. Interleukin 1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes, and related leukocyte cell lines. *J Clin Invest* 76: 2003–2011, 1985.
12. Blackman BR, Garcia–Cardena G, and Gimbrone MA Jr. A new in vitro model to evaluate differential responses of endothelial cells to simulated arterial shear stress waveforms. *J Biomech Eng* 124: 397–407, 2002.
13. Brookes PS. Mitochondrial nitric oxide synthase. *Mitochondrion*. 3: 187–204, 2004.
14. Chavez–Negrete A, Oropeza MV, Rojas MM, Villanueva T, and Campos MG. Starch-hemoglobin induces contraction on isolated rat aortic rings. *Artif Cells Blood Substit Immobil Biotechnol* 32: 549–561, 2004.
15. Cheng AM, Moore EE, Johnson JL, Walsh MD, Ao L, Moore PK, and Banerjee A. Polymerized hemoglobin induces heme oxygenase-1 protein expression and inhibits intercellular adhesion molecule-1 protein expression in human lung microvascular endothelial cells. *J Am Coll Surg* 201: 579–584, 2005.
16. Comair YG, Schipper HM, and Brem S. The prevention of oxyhemoglobin-induced endothelial and smooth muscle cytoskeletal injury by deferoxamine. *Neurosurgery* 32: 58–65, 1993.
17. D'Agnillo F and Alayash AI. Interactions of hemoglobin with hydrogen peroxide alters thiol levels and course of endothelial cell death. *Am J Physiol Heart Circ Physiol* 279: H1880–1889, 2000.
18. D'Agnillo F, Wood F, Porras C, Macdonald VW, and Alayash AI. Effects of hypoxia and glutathione depletion on hemoglobin- and myoglobin-mediated oxidative stress toward endothelium. *Biochim Biophys Acta* 1495: 150–159, 2000.
19. D'Agnillo F and Alayash AI. Redox cycling of diasparin cross-linked hemoglobin induces G2/M arrest and apoptosis in cultured endothelial cells. *Blood* 98: 3315–3323, 2001.
20. Dalziel K and O'Brien JR. Side reactions in the deoxygenation of dilute oxyhaemoglobin solutions by sodium dithionite. *Biochem J* 67: 119–124, 1957.
21. Dedkova EN, Ji X, Lipsius SL, and Blatter LA. Mitochondrial calcium uptake stimulates nitric oxide production in mitochondria of bovine vascular endothelial cells. *Am J Physiol Cell Physiol* 286: 406–415, 2004.
22. Donnelly LE and Barnes PJ. Expression of heme oxygenase in human airway epithelial cells. *Am J Respir Cell Mol Biol* 24: 295–303, 2001.
23. Duc G and Engel K. Effect of 2,3-DPG concentration on hemoglobin-oxygen affinity of whole blood. *Scand J Clin Lab Invest* 24: 405–412, 1969.
24. Dull RO, DeWitt BJ, Dinavahi R, Schwartz L, Hubert C, Pace N, and Fronticelli C. Quantitative assessment of hemoglobin-induced endothelial barrier dysfunction. *J Appl Physiol* 97: 1930–1937, 2004.
25. Everse J and Hsia N. The toxicities of native and modified hemoglobins. *Free Radic Biol Med* 22: 1075–1099, 1997.
26. Faivre–Fiorina B, Caron A, Fassot C, Fries I, Menu P, Labrude P, and Vigneron C. Presence of hemoglobin inside aortic endothelial cells after cell-free hemoglobin administration in guinea pigs. *Am J Physiol* 276: H766–H770, 1999.
27. Fitzpatrick CM, Savage SA, Kerby JD, Clouse WD, and Kashyap VS. Resuscitation with a blood substitute causes vasoconstriction without nitric oxide scavenging in a model of arterial hemorrhage. *J Am Coll Surg* 199: 693–701, 2004.
28. Gaucher C, Devaux C, Boura C, Lacolley P, Stoltz JF, and Menu P. *In vitro* impact of physiological shear stress on endothelial cells gene expression profile. *Clin Hemorheol Microcirc* 37: 99–107, 2007.
29. Geller DA, Nussler AK, Di Silvio M, Lowenstein CJ, Shapiro RA, Wang SC, Simmons RL, and Billiar TR. Cytokines, endotoxin, and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. *Proc Natl Acad Sci USA* 90: 522–526, 1993.
30. Giardina B, Messana I, Scatena R, and Castagnola M. The multiple functions of hemoglobin. *Crit Rev Biochem Mol Biol* 30: 165–196, 1995.
31. Giulivi C and Davies KJA. Hydrogen peroxide-mediated ferryl-hemoglobin generation in vitro and in red blood cells. *Methods Enzymol* 231: 490–496, 1994.
32. Giulivi C and Cadenas E. Heme protein radicals: formation, fate, and biological consequences. *Free Radic Biol Med* 24: 269–279, 1998.
33. Goldman DW, Breyer RJ 3rd, Yeh D, Brockner–Ryan BA, and Alayash AI. Acellular hemoglobin-mediated oxidative stress toward endothelium: a role for ferryl iron. *Am J Physiol* 275: H1046–1053, 1998.
34. Gruetter CA, Barry BK, McNamara DB, Gruetter DY, Kadowitz PJ, and Ignarro L. Relaxation of bovine coronary artery and activation of coronary arterial guanylate cyclase by nitric oxide, nitroprusside and a carcinogenic nitrosoamine. *J Cyclic Nucleotide Res* 5: 211–224, 1979.
35. Guidance for industry, criteria for safety and efficacity evaluation of oxygen therapeutics as red blood cells substitutes. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Biolgics Evaluation and Research. October 2004. http://www.fda.gov/cber/gdlns/oxytherbld.htm
36. Gutteridge JM and Smith A. Antioxidant protection by haemopexin of haem-stimulated lipid peroxidation. *Biochem J* 256: 861–865, 1988.
37. Halliwell B and Gutteridge JM. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* 219: 1–14, 1984.
38. Hess JR, MacDonald VW, and Brinkley WW. Systemic and pulmonary hypertension after resuscitation with cell-free hemoglobin. *J Appl Physiol* 74: 1769–78, 1993.
39. Howells G, Pham P, Taylor D, Foxwell B, and Feldmann M. Interleukin 4 induces interleukin 6 production by endothelial cells: synergy with interferon-gamma. *Eur J Immunol* 21: 97–101, 1991.
40. Huang Y, Yonetani T, Tsuneshige A, Hoffman BM, and Ackers GK. Heterometallic hybrids of homometallic human hemoglobins. *Proc Natl Acad Sci USA* 93: 4425–4430, 1996.
41. Ignarro LJ, Buga GM, Wood KS, Byrns RE, and Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci USA*, 84: 9265–9269, 1987.

42. Keilin D and Hartree EF. Reaction of methaemoglobin with hydrogen peroxide. *Nature* 166: 513–514, 1950.
43. Kellett GL and Schachman HK. Dissociation of hemoglobin into subunits. Monomer formation and the influence of ligands. *J Mol Biol* 59: 387–399, 1971.
44. Kellogg EW 3rd and Fridovich I. Superoxide, hydrogen peroxide, and singlet oxygen in lipid peroxidation by a xanthine oxidase system. *J Biol Chem* 250: 8812–8817, 1975.
45. Keyse SM, Applegate LA, Tromvoukis Y, and Tyrrell RM. Oxidant stress leads to transcriptional activation of the human heme oxygenase gene in cultured skin fibroblasts. *Mol Cell Biol* 10: 4967–4969, 1990.
46. Kleinbongard P, Schulz R, Rassaf T, Lauer T, Dejam A, Jax T, Kumara I, Gharini P, Kabanova S, Ozuyaman B, Schnurch HG, Godecke A, Weber AA, Robenek M, Robenek H, Bloch W, Rosen P, and Kelm M. Red blood cells express a functional endothelial nitric oxide synthase. *Blood* 107: 2943–2951, 2006.
47. Kobzik L, Stringer B, Balligand JL, Reid MB, and Stamler JS. Endothelial type nitric oxide synthase in skeletal muscle fibers: mitochondrial relationships. *Biochem Biophys Res Commun* 211: 375–381, 1995.
48. Kumar S and Bandyopadhyay U. Free heme toxicity and its detoxification systems in human. *Toxicol Lett* 157: 175–188, 2005.
49. Liu X and Spolarics Z. Methemoglobin is a potent activator of endothelial cells by stimulating IL-6 and IL-8 production and E-selectin membrane expression. *Am J Physiol Cell Physiol* 285: C1036–1046, 2003.
50. Lo SK, Janakidevi K, Lai L, and Malik AB. Hydrogen peroxide-induced increase in endothelial adhesiveness is dependent on ICAM-1 activation. *Am J Physiol* 264: L406–412, 1993.
51. Loppnow H and Libby P. Adult human vascular endothelial cells express the IL6 gene differentially in response to LPS or IL1. *Cell Immunol* 122: 493–503, 1989.
52. Marden MC, Kiger L, Poyart C, Rashid AK, Kister J, Stetzkowski–Marden F, Caron G, Haque M, and Moens L. Modulation of the oxygen affinity of cobalt-porphyrin by globin. *FEBS Lett* 472: 221–224, 2000.
53. Marini MA, Moore GL, Christensen SM, Fishman RM, Jessee RG, Medina F, Snell SM, and Zegna AI. Reexamination of the polymerization of pyridoxylated hemoglobin with glutaraldehyde. *Biopolymers* 29: 871–882, 1990.
54. McCoubrey WK Jr, TJ Huang, and MD Maines. Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. *Eur J Biochem* 247: 725–732, 1997.
55. McFaul SJ, Bowman PD, and Villa VM. Hemoglobin stimulates the release of proinflammatory cytokines from leukocytes in whole blood. *J Lab Clin Med* 135: 263–9, 2000.
56. McLeod LL and Alayash AI. Detection of a ferrylhemoglobin intermediate in an endothelial cell model after hypoxia-reoxygenation. *Am J Physiol* 277: H92–99, 1999.
57. Morrison M and Schonbaum GR. Peroxidase-catalyzed halogenation. *Annu Rev Biochem* 45: 861–888, 1976.
58. Motterlini R, Foresti R, Vandegriff K, Intaglietta M, and Winslow RM. Oxidative-stress response in vascular endothelial cells exposed to acellular hemoglobin solutions. *Am J Physiol* 269: H648–655, 1995.
59. Muller S, Legrand S, Wang X, Waché P, and Stoltz J–F. Actin filaments and shear stress in vascular endothelial cells. *Prob Fluid Mech Hydrol* 1: 299–306, 1999.
60. Nakai K, Ohta T, Sakuma I, Akama K, Kobayashi Y, Tokuyama S, Kitabatake A, Nakazato Y, Takahashi TA, and Sadayoshi S. Inhibition of endothelium-dependent relaxation by hemoglobin in rabbit aortic strips: comparison between acellular hemoglobin derivatives and cellular hemoglobins. *J Cardiovasc Pharmacol* 28: 115–123, 1996.
61. Nakai K, Sakuma I, Ohta T, Ando J, Kitabatake A, Nakazato Y, and Takahashi T. A. Permeability characteristics of hemoglobin derivatives across cultured endothelial cell monolayers. *J Lab Clin Med* 132: 313–319, 1998.
62. Nath KA, Haggard JJ, Croatt AJ, Grande JP, Poss KD, and Alam J. The indispensability of heme oxygenase-1 in protecting against acute heme protein-induced toxicity *in vivo*. *Am J Pathol* 156: 1527–1535, 2000.
63. Nathan C. Perspectives Series: Nitric oxide and nitric oxide synthases inducible nitric oxide synthase: what difference does it make? *J Clin Invest* 100: 2417–2423, 1997.
64. Okayama N, Park JH, Coe L, Granger DN, Ma L, Hisa CJ, and Alexander JS. Polynitroxyl alpha alpha-hemoglobin (PNH) inhibits peroxide and superoxide-mediated neutrophil adherence to human endothelial cells. *Free Radic Res* 31: 53–58, 1999.
65. Ozawa N, Shichiri M, Iwashina M, Fukai N, Yoshimoto T, and Hirata Y. Laminar shear stress up-regulates inducible nitric oxide synthase in the endothelium. *Hypertens Res* 27: 93–99, 2004.
66. Paller MS and Jacob HS. Cytochrome P-450 mediates tissue-damaging hydroxyl radical formation during reoxygenation of the kidney. *Proc Natl Acad Sci USA* 91: 7002–7006, 1994.
67. Palmer RM, Ferrige AG, and Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327: 524–526, 1987.
68. Pannen BH, Kohler N, Hole B, Bauer M, Clemens MG,and Geiger KK. Protective role of endogenous carbon monoxide in hepatic microcirculatory dysfunction after hemorrhagic shock in rats. *J Clin Invest* 102: 1220–1228, 1998.
69. Patel RP, Svistunenko DA, Darley–Usmar VM, Symons MC, and Wilson MT. Redox cycling of human methaemoglobin by H2O2 yields persistent ferryl iron and protein based radicals. *Free Radic Res* 25: 117–123, 1996.
70. Patel RP. Biochemical aspects of the reaction of hemoglobin and NO: implications for Hb-based blood substitutes. *Free Radic Biol Med* 28: 1518–1525, 2000.
71. Pober JS. Effects of tumour necrosis factor and related cytokines on vascular endothelial cells. *Ciba Found Symp* 131: 170–184, 1987.
72. Pollock JS, Nakane M, Buttery LD, Martinez A, Springall D, Polak JM, Forstermann U, and Murad F. Characterization and localization of endothelial nitric oxide synthase using specific monoclonal antibodies. *Am J Physiol* 265: C1379–1387, 1993.
73. Ponka P. Cell biology of heme. *Am J Med Sci* 318: 241–256, 1999.
74. Rees DD, Palmer RM, Schulz R, Hodson HF, and Moncada S. Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br J Pharmacol* 101: 746–752, 1990.
75. Savitsky JP, Doczi J, Black J, and Arnold JD. A clinical safety trial of stroma-free hemoglobin. *Clin Pharmacol Ther* 23: 73–80, 1978.
76. Shibahara S, Yoshizawa M, Suzuki H, Takeda K, Meguro K, and Endo K. Functional analysis of cDNAs for two types of human heme oxygenase and evidence for their separate regulation. *J Biochem* 13: 214–218, 1993.
77. Simoni J, Simoni G, Lox CD, and Feola M. Reaction of human endothelial cells to bovine hemoglobin solutions and tumor necrosis factor. *Artif Cells Blood Substit Immobil Biotechnol* 22: 777–787, 1994.
78. Simoni J, Simoni G, Martinez–Zaguilan R, Wesson DE, Lox CD, Prien SD, and Kumar RV. Improved blood substitute: evaluation of its effects on human endothelial cells. *ASAIO J* 44: M356–367, 1998.
79. Svistunenko DA, Patel RP, Voloshchenko SV, and Wilson MT. The globin-based free radical of ferryl hemoglobin is detected in normal human blood. *J Biol Chem* 272: 7114–7121, 1997.
80. Tenhunen, R., Marver, H. S., and Schmid, R. (1969). Microsomal heme oxygenase. Characterization of the enzyme. *J Biol Chem* 244: 6388-6394, 1969.
81. Toussaint–Hacquard M, Devaux Y, Longrois D, Faivre–Fiorina B, Muller S, Stoltz J.F, Vigneron C, and Menu P. Biological response of human aortic endothelial cells exposed to acellular hemoglobin solutions developed as potential blood substitutes. *Life Sci* 72: 1143–1157, 2003.
82. Tsai AG, Cabrales P, Manjula BN, Acharya SA, Winslow RM, and Intaglietta M. Dissociation of local nitric oxide concentration and vasoconstriction in the presence of cell-free hemoglobin oxygen carriers. *Blood* 108: 3603–3610, 2006.
83. Ungureanu–Longrois D, Balligand JL, Okada I, Simmons WW, Kobzik L, Lowenstein CJ, Kunkel SL, Michel T, Kelly RA, and Smith TW. Contractile responsiveness of ventricular myocytes to

isoproterenol is regulated by induction of nitric oxide synthase activity in cardiac microvascular endothelial cells in heterotypic primary culture. *Circ Res* 77: 486–493, 1995.
84. van Kooten TG, Schakenraad JM, Van der Mei HC, and Busscher HJ. Development and use of a parallel-plate flow chamber for studying cellular adhesion to solid surfaces. *J Biomed Mater Res* 26: 725–38, 1992.
85. Venkateshrao S, Venkatesh B, and Manoharan PT. Studies on nitrosyl hemes in Ni(II)-Fe(II) hybrid hemoglobins. *Nitric Oxide* 13: 226–231, 2005.
86. Vo PA, Reid JJ, and Rand MJ. Attenuation of vasoconstriction by endogenous nitric oxide in rat caudal artery. *Br J Pharmacol* 107: 1121–1128, 1992.
87. Vogel WM, Dennis RC, Cassidy G, Apstein CS, and Valeri CR. Coronary constrictor effect of stroma-free hemoglobin solutions. *Am J Physiol* 251: H413–420, 1986.
88. Wagener, Feldman E, de Witte T, and Abraham NG. Heme induces the expression of adhesion molecules ICAM-1, VCAM-1, and E selectin in vascular endothelial cells. *Proc Soc Exp Biol Med* 216: 456–463, 1997.
89. Wagener FA, da Silva JL, Farley T, de Witte T, Kappas A, and Abraham NG. Differential effects of heme oxygenase isoforms on heme mediation of endothelial intracellular adhesion molecule 1 expression. *J Pharmacol Exp Ther* 291: 416–423, 1999.
90. Wakabayashi Y, Takamiya R, Mizuki A, Kyokane T, Goda N, Yamaguchi T, Takeoka S, Tsuchida E, Suematsu M, and Ishimura Y. Carbon monoxide overproduced by heme oxygenase-1 causes a reduction of vascular resistance in perfused rat liver. *Am J Physiol* 277: G1088–1096, 1999.
91. Wallis JP. Nitric oxide and blood: a review. *Transfus Med* 15: 1–11, 2005.
92. Weiss SJ. Tissue destruction by neutrophils. *N Engl J Med* 320: 365–376, 1989.
93. Winslow RM. Current status of oxygen carriers ('blood substitutes') *Vox Sang*. 91: 102–110, 2006.
94. Xie QW, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Ding A, Troso T, and Nathan C. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science*. 256: 225–228, 1992.
95. Yeh LH and Alayash AI. Effects of cell-free hemoglobin on hypoxia-inducible factor (HIF-1alpha) and heme oxygenase (HO-1) expressions in endothelial cells subjected to hypoxia. *Antioxid Redox Signal* 6: 944–953, 2004.
96. Yoshida T, Biro P, Cohen T, Muller RM, and Shibahara S. Human heme oxygenase cDNA and induction of its mRNA by hemin. *Eur J Biochem* 171: 457–461, 1998.
97. Zhang H, Weir BK, Macdonald RL, Marton LS, Solenski NJ, Kwan AL, and Lee KS. Mechanisms of $[Ca^{++}]i$ elevation induced by erythrocyte components in endothelial cells. *J Pharmacol Exp Ther* 277: 1501–1509, 1996.
98. Zhu X–L, Gafney WCM, Pacheco ND, and Rollwagen FM. Kinetics of cytokine gene expression in macrophage and endothelial cell lines following liposome encapsulated haemoglobin (LEH) treatment *in vitro*. *Cytokine* 8: 541–547, 1996.
99. Ziegler T, Bouzourene K, Harrison VJ, Brunner HR, and Hayoz D. Influence of oscillatory and unidirectional flow environments on the expression of endothelin and nitric oxide synthase in cultured endothelial cells. *Arterioscler Thromb Vasc Biol* 18: 686–692, 1998.
100. Zulueta JJ, Sawhney R, Yu FS, Cote CC, and Hassoun PM. Intracellular generation of reactive oxygen species in endothelial cells exposed to anoxia-reoxygenation. *Am J Physiol* 272: L897–902, 1997.

Address reprint requests to:
Dr. Patrick Menu
Groupe Mécanique et Ingénierie Cellulaire et Tissulaire
LEMTA-UMR 7563 IFR 111
Faculté de Médecine
Vandoeuvre-lès-Nancy, France

E-mail: patrick.menu@pharma.uhp-nancy.fr

Date of first submission to ARS Central, November 22, 2007; date of final revised submission, December 21, 2007; date of acceptance, December 25, 2007.

This article has been cited by: