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The importance of the effect of shear stress on endothelial cells in determining the performance of hemoglobin based oxygen carriers

Caroline Gaucher-Di Stasio [a], Estelle Paternotte [a], Christine Prin-Mathieu [c], Brandon J. Reeder [d], Gaël Poitevin [b], Pierre Labrude [a], Jean-François Stoltz [a], Chris E. Cooper [d], Patrick Menu [a,*]

[a] Groupe Ingénierie Cellulaire et Tissulaire, LEMTA-UMR 7563 IFR 111, Faculté de médecine, Avenue de la Forêt de Haye, Vandoeuvre-lès-Nancy, France
[b] Unité mixte UHP-INSERM U684, Faculté de Médecine, Vandoeuvre-lès-Nancy, France
[c] Laboratoire d'immunologie, Faculté de Médecine, CHU Nancy, Vandoeuvre-lès-Nancy, France
[d] Department of Biological Sciences, University of Essex, Wivenhoe Park, Colchester, Essex CO4 3SQ, UK

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ABSTRACT

The lack of blood donations and the threat of infections from blood and blood products have led to extensive research into the development of blood substitutes. The latest generation of hemoglobin based oxygen carriers (HBOC) has been shown to induce side effects like hypertension, vasoconstriction, inflammation and oxidative stress. HBOC are able to restore volemia and transport oxygen after a hemorrhagic shock, the reperfusion leading to the restoration of the blood flow in vessels. We propose an innovative approach, more closely emulating clinical situations, to assess the impact of HBOC perfusion on endothelial cells (EC) in vitro. Through this approach we quantified levels of oxidative stress, vasoactive factors and inflammation. EC were cultivated under a laminar flow to reproduce the return of shear stress (SS) during the reperfusion. We showed that heme oxygenase I transcription correlated with changes in oxidatively modified heme and methemoglobin; all were lower under SS. SS induced increased nitric oxide production, which may have implications for the mechanism of in vivo vasoconstriction and hypertension. E-selectin changes under SS were greater than those of ICAM-1. Our results demonstrate how it is essential to include SS in assays attempting to understand the potential vascular side effects of HBOC perfusion.

1. Introduction

Blood from donors is a limited resource and its transfusion is associated with significant adverse effects; hence there is great interest in the development of blood substitutes and oxygen carrying compounds. Different categories of blood substitutes are available today; these include plasma expanders, perfluorocarbon emulsions (PFC) and hemoglobin based oxygen carriers (HBOC). Different chemically modified HBOC, obtained from natural sources or using recombinant technology, are currently under clinical investigations. Chemical modifications including polymerization, cross-linking, conjugation with macromolecules (polyethylene glycol (PEG) or dextran), and encapsulation into phospholipids vesicles have been used to increase hemoglobin (Hb) vascular half-life and oxygen release properties [1]. Although blood substitutes have been extensively studied, side reactions still persist that have limited their current clinical usefulness [2].

When injected in the bloodstream, HBOC are in direct contact with endothelial cells (EC). There is limited information available in the literature on in vitro interactions of Hb solutions with EC; however, problems with oxidative stress, vasomotion and inflammation have been highlighted [3]. Oxidative stress can be induced by Hb autooxidation, forming methemoglobin (metHb) in the presence of oxygen. This process can lead to the formation of reactive oxygen species (ROS) [4,5]. ROS generated by autooxidation can lead to oxidative reactions on the Hb molecule itself, including formation of ferrylHb and free radicals. FerrylHb and MetHb cannot transport O_2 and FerrylHb is cytotoxic [4]. Because heme release and heme autooxidation occur naturally in the blood when Hb leaks out of the red blood cells, EC contain a protective system of detoxification including heme oxygenase (HO) [6]. One of the major consequences of increased vascular ROS production is

Abbreviations: Dex-BTC-Hb, dextran-benzene tetracarboxylate-hemoglobin; EC, endothelial cells; eNOS/iNOS, endothelial/inducible NO synthase; Hb, hemoglobin; HBOC, hemoglobin based oxygen carriers; HES, hydroxyethyl starch; HO-1, heme oxygenase-1; ICAM-1, intercellular adhesion molecule; MetHb, methemoglobin; NO, nitric oxide; OxHm, oxidatively modified heme; PEG, polyethylene glycol; PFC, perfluorocarbon; REL, relative expression level; ROS, reactive oxygen species; SS, shear stress; SSRE, shear stress response element.

* Corresponding author. Tel.: +33 3 83 68 34 60; fax: +33 3 83 68 34 59.
E-mail address: patrick.menu@medecine.uhp-nancy.fr (P. Menu).

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a reduction of endothelial nitric oxide (NO) bioavailability [7]. This reduction of NO bioavailability has been implicated in the mechanism of vasoconstriction observed in vivo after the administration of HBOC [8–10]. Indeed, Hb is known to scavenge NO, decreasing its bioavailability for smooth muscle cells [11]. Both NO synthesis and eNOS transcriptional regulation are of interest as an increase in eNOS transcription could counter NO depletion in the circulation. Finally, HBOC are able to induce inflammation due to their exogenous origin. The endothelium has been implicated in leukocytes diapedesis by InterCellular Adhesion Molecule-1 (ICAM-1) and E-selectin membrane expression. ICAM-1 has been extensively investigated to highlight the inflammatory effect of HBOC on EC [12–15] whereas E-selectin has not been investigated yet. A study of both of them seems to be imperative because a change in the early (E-selectin) and later (ICAM-1) stages of diapedesis can transform the inflammatory response of EC faced with Hb.

Vascular EC are constantly subjected to shear stress (SS) imposed by blood flow. The application of SS to EC activates a number of mechanosensors. This mechanosensing regulates the functional behavior of EC under healthy and disease conditions. Mechanosensing has been implicated in the stimulation of genes containing a SS response element (SSRE) in their promoter, such as ICAM-1 [16] and eNOS [17]. Reperfusion after a large hemorrhage restores SS to the organism. Therefore, both the introduction of HBOC and the return of laminar flow onto EC can act in synergy to amplify or minimize phenomena observed during reperfusion.

In this study, we propose a novel method that closely represents physiological conditions under in vitro conditions, to assess the impact of blood substitutes on EC. We have tested two different HBOC, compared to a plasma expander, to assess oxidative stress, the regulation of vasoactive factors and the inflammatory responses of EC. We demonstrate that SS is an essential parameter that influences the interaction of HBOC and EC. Our results and our novel strategy are a new way to screen blood substitutes or other pharmacological molecules more effectively under in vitro conditions to limit animal use during experimental studies.

2. Materials and methods

2.1. Blood substitutes

Two modified Hb solutions were studied (Table 1) and prepared as follows: Dex-BTC-Hb was made by conjugating human Hb to dextran–benzene-tetracarboxylate macromolecules and was obtained from Pasteur Mérieux Serums et Vaccins. This conjugation increases oxygen half-saturation pressure (P_{50}) and vascular half-life [18]. Oxyglobin® a glutaraldehyde-polymerized bovine Hb (Biopure Corporation, USA), was provided courtesy of Dr. A. Alayash (Center for Biologics Evaluation, Food and Drug Administration, Bethesda, MD, USA). These Hb solutions were diluted at 16 g/L in culture medium. This value corresponds to the plasmatic Hb concentration after a 1/5 volemic compensation of the total blood volume with an 80 g/L HBOC solution. This Hb concentration in the plasma simulates levels observed in clinical situations of hemodilution, such as cardiopulmonary bypass or resuscitation from hypovolemic shock. Controls used were Voluven® 6% (w/v) (Fresenius Kabi, France) diluted at 1/5 in culture medium or culture medium alone. Voluven® is a 130-kDa hydroxyethyl starch (HES) that exhibits an oncotic pressure close to the HBOC studied.

2.2. Cell culture and seeding

Human umbilical vein EC (HUVEC) were isolated from three different donors for each experiment according to the method of Jaffe and coworkers [19]. HUVEC were cultured at 37 °C, 5% CO_2 in 25 cm^2 tissue-culture-treated flask in complete medium. The medium consisted of an equal mixture of M199 and RPMI1640 without phenol red, supplemented with 20% (v/v) pooled human serum, 2 mmol/L L-glutamin, 20 mmol/L HEPES, 100 IU/mL penicillin, 100 mg/mL streptomycin and 2.5 mg/mL Fungizon®. HUVEC, at the first culture passage, were seeded for 48 h, at 10 000 cells/cm^2 on glass slides coated with 1% gelatin (Sigma, France).

2.3. Flow system

A rectangle parallel flow chamber was used, equipped with one silicone gasket 0.024 cm thickness containing a rectangular opening of 1.8 cm width and 3.2 cm length. Glass slides seeded with cells were assembled with parallel flow chambers by vacuum (about −450 mbar). A steady flow rate was generated by a peristaltic pump (Masterflex®, Cole Parmer, USA), and HUVEC were submitted to laminar SS for 6 h at 1 Pa at 37 °C oxygenated at 5% CO_2. The flow rate was adapted to the viscosity of blood substitutes in order to apply 1 Pa whatever are the molecules used.

Slides seeded with HUVEC placed in an incubator at 37 °C with 5% CO_2 were used as static controls.

2.4. Measurement of metHb formation

MetHb levels before and after experiments were quantified spectrophotometrically between 450 and 700 nm. Microsoft Excel solver was used to minimize the least squares differences between the experimental spectrum and a mixture of pure oxy and metHb spectra. An analysis of the residuals indicated that no significant concentrations of other Hb derivatives were present to interfere with the quantitation.

2.5. Measurement of NO metabolites

The sum of NO, nitrite and nitrate was quantified in cell supernatants using the commercially available Total NO/Nitrite/Nitrate kit (R&D systems, France). To eliminate protein interference with the assay, the cell culture supernatants were subjected to two ultrafiltrations with 50 kDa and 10 kDa microcon (Millipore, France). Concentrations in μM were expressed for 10^6 cells.

2.6. Quantitative real-time PCR (qPCR)

Total RNA from HUVEC was extracted with a combination of the TRIzol® reagent (Invitrogen, France) for lysis and the RNeasy minikit (Qiagen, France). One microgram total RNA was reverse transcribed into cDNA using iScript™ cDNA Synthesis kit (Bio-rad, France) according to the manufacturer's instructions.

qPCR was performed using the SYBR Green qPCR kit (Bio-rad, France) in the iCycler real-time PCR detection system (Bio-rad, France) according to the manufacturer's instructions. RPS29 was used as an internal control to normalize gene amplification results. Gene expression analysis was performed using the iCycler® iQ Real-Time PCR Detection System 1.0 (Bio-rad, France).

2.7. Quantification of ICAM-1 and E-selectin sites on EC surface

EC were detached from glass slides with 0.05% trypsin/EDTA (Sigma, France). Cells were incubated for 30 min at room temperature with 1/50 of monoclonal ICAM-1 or E-selectin antibody (Santa Cruz, France) and then with 1/50 of the secondary antibody (Alexa Fluor® 488 goat anti-rabbit IgG, Molecular Probes, France). Finally, cells were fixed with 300 μL of 1% paraformaldehyde for 10 min. Fluorescent intensities were measured with Cytomics FC500 (Beckman Coulter, France). The quantification of ICAM-1 and E-selectin number of sites was performed using the Dako Qifikit (Dako, France).

Table 1
Physical and chemical properties of blood substitutes. All these data are provided by manufacturers of the different solutions.

	Voluven®	Dex-BTC-Hb	Oxyglobin®
Molecule	Hydroxyethyl starch (HES)	Human Hb	Bovine Hb
Concentration	6% (w/v)	85 g/L	130 g/L
Solution dilution	Sodium chloride	Saccharose	Modified Ringer lactate
Mean molecular weight (kDa)	130	200	100
P_{50}[a] (Torr)	[b]	23	27
pH	4–5.5	7.2	7.8
Endotoxins (UE/mL)	<0.05	<0.5	<0.05

[a] P_{50}, Oxygen half-saturation pressure of Hb measured in standard conditions.
[b] Not concerned.