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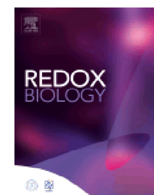

Research Paper

Absorbance and redox based approaches for measuring free heme and free hemoglobin in biological matrices

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ABSTRACT

Cell-free heme (CFH) and hemoglobin (Hb) have emerged as distinct mediators of acute injury characterized by inflammation and microcirculatory dysfunction in hemolytic conditions and critical illness. Several reports have shown changes in Hb and CFH in specific pathophysiological settings. Using PBS, plasma from patients with sickle cell disease, acute respiratory distress syndrome (ARDS) patients and supernatants from red cells units, we found that commonly used assays and commercially available kits do not distinguish between CFH and Hb. Furthermore, they suffer from a variety of false-positive interferences and limitations (including from bilirubin) that lead to either over- or underestimation of CFH and/or Hb. Moreover, commonly used protocols to separate CFH and Hb based on molecular weight (MWt) are inefficient due to CFH hydrophobicity. In this study, we developed and validated a new approach based on absorbance spectrum deconvolution with least square fitting analyses that overcomes these limitations and simultaneously measures CFH and Hb in simple aqueous buffers, plasma or when associated with red cell derived microvesicles. We show how incorporating other plasma factors that absorb light over the visible wavelength range (specifically bilirubin), coupled with truncating the wavelength range analyzed, or addition of mild detergent significantly improves fits allowing measurement of oxyHb, CFH and metHb with $>90\%$ accuracy. When this approach was applied to samples from SCD patients, we observed that CFH levels are higher than previously reported and of similar magnitude to Hb.

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1. Introduction

Cell free heme (plasma free heme + plasma protein bound heme) (CFH) and cell free hemoglobin (Hb), are mediators of tissue injury in hemolytic diseases (e.g. sickle cell disease (SCD), transfusion toxicity, cardiopulmonary bypass, dialysis, infection [1–14]) as well as other diseases not typically associated with hemolysis including environmental poisons and sepsis [15–21]. Cell-free Hb is a potent scavenger of nitric oxide (NO), activates inflammation and undergoes redox cycling reactions that cause oxidative stress [1,8,22–25]. Cell-free heme also stimulates oxidative stress, activates TLR4, and the inflammasome leading to exacerbated inflammation-mediated tissue injury [7,9,19,26–29]. Thus, CFH and Hb elicit tissue injury by overlapping and distinct mechanisms.

Because of the implication that CFH and Hb levels may have on disease mechanisms and therapeutics, it is imperative that methods used to measure each are accurate, sensitive and reproducible. In vivo, CFH and Hb will co-exist and need to be distinguished

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from each other when quantifying. Several methods have been used to measure CFH and Hb in biological matrices. However, these typically rely on a colorimetric detection of CFH and Hb under conditions that may cause Hb denaturation and heme release. Therefore they are unlikely to distinguish between CFH and Hb. Other approaches that improve sensitivity utilize the pseudo-peroxidase activity of heme, and link heme to a reporter molecule whose oxidation can be detected by a change in absorbance or fluorescence. Again, these approaches would not distinguish between CFH and Hb, as both are redox active. Our assessment of the literature is that these considerations are often overlooked when measuring CFH and Hb. When appreciated, separation methods based on differing size of CFH and Hb are employed (e.g. Centricon filtration devices). However, these are typically not optimized for separation of hydrophobic compounds such as CFH.

To our knowledge, no systematic study comparing different CFH and Hb measurement protocols have been reported. In this study, we demonstrate that commonly used methods to measure CFH and Hb do not distinguish between them, presenting a problem when both are present in the same medium. We develop a method based on spectral deconvolution, allowing for simultaneous and rapid measurement of CFH and Hb in simple aqueous systems and plasma. *Pros* and *cons* are discussed and applicability to evaluate CFH and Hb in clinical samples demonstrated.

2. Materials and methods

2.1. Materials

All chemicals, reagents and assay kits were purchased from Sigma unless otherwise noted.

2.2. Plasma preparation

Blood was collected by venipuncture from healthy volunteers per UAB IRB approved protocols, and centrifuged 1500 × *g*, 4 °C, 10 min to pellet red blood cells (RBCs). Plasma was collected, stored on ice and used within 24 h.

2.3. Trauma-hemorrhage patient's sample collection

Blood was collected from 37 resuscitated trauma patients according to UAB IRB approved protocols. Blood was centrifuged within 60 min of collection and plasma stored at 4 °C for up to 72 h before freezing in liquid nitrogen. Samples were stored at −80 °C and subsequently thawed on ice for spectral measurements.

2.4. Critically ill patients' sample collection

Plasma was collected from patients with ARDS secondary to Gram negative sepsis and from other critical non-pulmonary conditions, who were intubated and mechanically ventilated in the medical intensive care unit at UAB. All human studies were approved by the UAB Institutional Review Board.

2.5. Sickle cell disease (SCD) patient's sample collection

Within 24 h of admission, blood was collected from SCD patients per UAB IRB approved protocols and recollected every 24 h until discharge or until logistical constraints prevented collection. In order to be included, patients had to have homozygous Hb SS or Hb S Beta Thalassemia (Hb SB0), be 1–21 years old, and admitted for acute chest syndrome (ACS) or vaso-occlusive pain crisis (VOC). Control samples were collected from age-matched African American patients without SCD. Blood was collected in sodium citrate tubes, immediately placed on ice and centrifuged at 1500 rpm, 4 °C for 15 min, within 30 min of collection. Plasma was aliquoted and stored at 4 °C until the aliquots could be rapidly frozen using liquid nitrogen.

2.6. Stored RBC sampling and microvesicle preparation

Leukoreduced RBCs (from six distinct blood donors, with 2 bags collected from one donor) in Adsol were sampled from bags stored for 30–52d in the UAB Blood Bank. Bags were allocated for disposal or expired. RBC were centrifuged 3000 × *g*, 10 min, 4 °C to pellet RBC and provide supernatants. The latter were further processed to isolate microparticles (MP) and exosomes (Exo) by two centrifugations at 10,000 × *g*, 30 min, 4 °C (spin 2 and 3) followed by a 150,000 × *g*, 2 h, 4 °C (spin 4) centrifugation. Pellets from spins 2 and 3 were combined to give MPs, and pellet from spin 4 contained exosomes. Microvesicle size and number were determined using an NS300 Nanosight (Malvern, UK) with an attached syringe sample pump and 405 nm laser.

2.7. Hemoglobin preparation

Cell-free oxyHb was purified from RBCs and catalase removed as described [30,31]. All hemoglobin was stored in the carbon-monoxide ligated form, and converted to oxyHb or metHb immediately prior to use as described [32]. For cyanide treatment, potassium cyanide (500 mM in 0.1 M KOH) was added to Hb or CFH resulting in cyanide: heme ratios >10; and pH was monitored to keep at 7.4. Final concentration of cyanide is indicated in text.

2.8. Cell-free heme (CFH) preparation

10 mM hemin (Frontier Scientific, UT) was prepared in 0.1 M NaOH, then diluted to 100 µM in PBS, pH 7.4 at 22 °C, fresh on the day of each experiment.

2.9. Conjugated and unconjugated bilirubin

Conjugated (Di-taurine conjugated) bilirubin and unconjugated bilirubin (Lee BioSolutions, MO) were prepared in PBS (2 mg/mL) and ethanol (1 mg/mL) respectively and diluted to indicated concentrations.

2.10. Quantifying CFH, OxyHb, MetHb, and CN-Hb

All CFH and Hb levels are reported in heme (i.e. 1 µM Hb tetramer will be 4 µM Heme expressed per Hb). Various protocols were used to quantify CFH and Hb including:

i) QuantiChrom™ Heme Assay Kit and QuantiChrom™ Hemoglobin Assay Kit (BioAssay, Hayward, CA). CFH and Hb levels were determined using manufacturer's protocols and calibration standards provided. Changes in absorbance at 405 nm were measured using a 96-well plate reader (Victor^3, Perkin Elmer, MA).
ii) CFH or Hb were measured using the TMB Substrate Reagent Set (BD Biosciences, San Jose, CA) with modifications to the manufacturer's protocol: 50 µL PBS or 50 µL diluted plasma (10–200x in PBS) were spiked with oxyHb or CFH to final concentrations of 0–2 µM. PBS or diluted plasma were then mixed with 150 µL of a solution containing hydrogen peroxide and TMB (prepared per manufacturer's instructions), all pre-equilibrated at 37 °C prior to mixing. TMB-oxidation was monitored continuously by following absorbance change at

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