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Ascorbic acid attenuates endothelial permeability triggered by cell-free hemoglobin

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Abstract

Background—Increased endothelial permeability is central to shock and organ dysfunction in sepsis but therapeutics targeted to known mediators of increased endothelial permeability have been unsuccessful in patient studies. We previously reported that cell-free hemoglobin (CFH) is elevated in the majority of patients with sepsis and is associated with organ dysfunction, poor clinical outcomes and elevated markers of oxidant injury. Others have shown that Vitamin C (ascorbate) may have endothelial protective effects in sepsis. In this study, we tested the hypothesis that high levels of CFH, as seen in the circulation of patients with sepsis, disrupt endothelial barrier integrity.

Methods—Human umbilical vein endothelial cells (HUVEC) were grown to confluence and treated with CFH with or without ascorbate. Monolayer permeability was measured by Electric Cell-substrate Impedance Sensing (ECIS) or transfer of ^{14}C-inulin. Viability was measured by trypan blue exclusion. Intracellular ascorbate was measured by HPLC.

Results—CFH increased permeability in a dose- and time-dependent manner with 1 mg/ml of CFH increasing inulin transfer by 50% without affecting cell viability. CFH (1 mg/ml) also caused a dramatic reduction in intracellular ascorbate in the same time frame (1.4 mM without CFH, 0.23 mM 18 hours after 1 mg/ml CFH, $p<0.05$). Pre-treatment of HUVECs with ascorbate attenuated CFH induced permeability.

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Conclusions—CFH increases endothelial permeability in part through depletion of intracellular ascorbate. Supplementation of ascorbate can attenuate increases in permeability mediated by CFH suggesting a possible therapeutic approach in sepsis.

Keywords

Cell-free hemoglobin; endothelial permeability; ascorbic acid; oxidative stress

INTRODUCTION

The integrity of the vascular endothelium is critical to the maintenance of homeostasis, yet is compromised in sepsis [1] leading to increased microvascular permeability, shock, and organ dysfunction [2]. A number of cellular and molecular mechanisms that regulate the endothelial barrier have been described including tight junction proteins, the actin cytoskeleton and the Rho kinase pathway [3]. However, the specific triggers of endothelial barrier breakdown of these homeostatic mechanisms in sepsis are largely unknown. Our group has identified cell-free hemoglobin (CFH), hemoglobin that has escaped the confines of the red blood cell, as one potential trigger of increased endothelial permeability in sepsis [4].

Hemoglobin consists of two alpha and two beta subunits, each containing an iron atom within a heme group. The iron atom usually exists in the ferrous (Fe^{2+}) state, but can become oxidized to ferric (Fe^{3+}) or ferryl (Fe^{4+}) forms in a pro-oxidant environment. Red blood cells become fragile during sepsis leading to hemolysis and release of hemoglobin. [5,6] Failure to scavenge cell-free hemoglobin (CFH) triggers inflammation, tissue damage, and endothelial dysfunction.[7] We have previously shown that circulating levels of CFH are elevated in 80% of sepsis patients [8] and are associated with organ dysfunction and mortality [4]. In many of these patients, ferryl hemoglobin is detected [9]. Patients with sepsis also have high plasma levels of isoprostanes, a product of lipid peroxidation that serves as a biomarkers of oxidative stress [10]. While it is well recognized that hemolysis and liberation of CFH is common in sepsis, the mechanisms underlying its pathogenic effects are not well understood.

Ascorbate (vitamin C) is a potent antioxidant that is critical for endothelial barrier maintenance during inflammation [11] [12]. In endothelial cells, intracellular ascorbate can tighten the endothelial barrier by increasing intracellular nitric oxide (NO) and cyclic GMP. However, during inflammatory states including sepsis and critical illness, plasma ascorbate levels decline [13,14], potentially leading to intracellular ascorbate depletion and increased vascular permeability. Another way ascorbate protects the endothelium is by reducing pro-oxidant molecules to a less injurious form. Reduction of pro-oxidant molecules depletes ascorbate stores and may contribute to end-organ injury. One of the potential targets of ascorbate during critical illness may be CFH as it is known that CFH, a potent oxidant, can be reduced by ascorbate [15]. In this manuscript, we test the hypothesis that high levels of CFH, as seen in the circulation of patients with sepsis, cause depletion of intracellular ascorbate in endothelial cells, thereby disrupting endothelial barrier function.

MATERIALS AND METHODS

Reagents

A fresh stock of CFH was prepared before each experiment by dissolving 10 mg endotoxin-free native human hemoglobin (Cell Sciences, Canton, MA) in 10 ml sterile 0.9% NaCl. A fresh stock of ascorbate was prepared before each experiment by dissolving L-ascorbic acid (Sigma, St. Louis, MO) in sterile H_2O. EDTA was purchased from Mediatech/CellGro-Corning (Manassas, VA). ^{14}C-inulin (MW ~ 5000–5500; 2 mCi/g) was purchased from Perkin Elmer Life Sciences (Waltham, WA).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from ScienCell Research Laboratories (Carlsbad, CA) or Lonza (Basel, Switzerland) and cultured in proprietary media from the respective suppliers in the absence of antioxidant supplements at 37°C in humidified air containing 5% CO_2. Cells were used between passages 1–5. Reagents were sterilized with a 0.22 μm filter prior to addition to cultured cells. For all experiments, cells were grown to confluence then incubated with CFH (0–1 mg/ml) with or without ascorbate (0–60 μM) for 18 hours.

Endothelial permeability

Electric Cell-substrate Impedance Sensing—HUVECs were cultured to confluence in 8-well arrays overlying electrodes according to the manufacturer's protocol (Applied Biophysics, Troy, NY). Alternating current was applied to each electrode at the bottom of each well and the voltage potential on the luminal side was detected. From these two measurements, impedance was calculated according to Ohm's Law. Resistance was then calculated and is directly correlated to barrier integrity; when the endothelial monolayer is compromised, resistance decreases.

Transfer of radiolabeled inulin—Cells were cultured to confluence as determined by light microscopy on polyethylene terephthalate cell culture inserts (6-well plates with 0.4 μm pores at a density of $2 \pm 0.2 \times 10^6$ pores per cm^2, Falcon BD Biosciences) with 1.7 ml antioxidant-free medium in the upper well and 2.8 ml antioxidant-free medium in the lower well. After several days of confluence to ensure development of a tight barrier, reagents were added as indicated. Transfer of ^{14}C-inulin from luminal to abluminal chambers over 60 min at 37°C was measured in duplicate as previously described.[16] The permeability coefficient of ^{14}C-inulin was calculated with correction for the rate of ^{14}C-inulin transfer across filters after removal of cells with ammonium hydroxide.

Intracellular ascorbate

Ascorbate was measured in cells cultured in 6-well plates following 3 rinses with Krebs-Ringer Hepes buffer (KRH, in mM: 20 Hepes, 128 NaCl, 5.2 KCl, 1 NaH_2PO_4, 1.4 $MgSO_4$, and 1.4 $CaCl_2$) at pH 7.4. The cell monolayer was then treated with 100 μL of 25% (w/v) metaphosphoric acid, lifted from the plate with a rubber spatula, and the acidic lysate was partially neutralized with 350 μL of 100 mM Na_2HPO_4 containing 50 μM EDTA, pH 8.0. The cell lysate was centrifuged at 4°C for 1 min at 13,000 *x g* and the supernatant was

collected for assay of ascorbate by high-performance liquid chromatography in duplicate as previously described [17,18]. Intracellular ascorbate concentrations were calculated as a fraction of the intracellular distribution space of 3-O-methylglucose relative to the cell protein content [19] which was taken as 3.6 ± 1.2 μL/mg protein [16].

Viability

Confluent HUVECs were incubated with CFH (0–1 mg/ml) for 18 h. Cell viability was determined by trypan blue exclusion, after washing with EDTA (1mM) to remove extracellular hemoglobin.

Data analyses

Data are expressed as means ± SEM. Determination of significant differences between all groups was done with one-way ANOVA and Tukey post hoc test or Mann-Whitney U, as specified. Significance was defined as $p < 0.05$.

RESULTS

Cell-free hemoglobin decreases endothelial monolayer electrical resistance

To test whether CFH disrupts endothelial barrier integrity, we measured electrical resistance of a HUVEC monolayer using Electric Cell-substrate Impedance Sensing (ECIS). An intact, tight endothelial barrier presents a high resistance to current flow. When the barrier is disrupted, current flows more easily and is registered as a drop in resistance. We found that CFH caused a time-dependent (Figure 1A) and dose-dependent (Figure 1B) decrease in electrical resistance, consistent with disruption of the endothelial barrier by CFH.

Cell-free hemoglobin increases endothelial macromolecular permeability

Decreased trans-endothelial resistance following CFH exposure reflects a loss of barrier function, but resistance measurements do not provide any information on the size of particles that might be able to cross the disrupted endothelium. We next tested the hypothesis that CFH would mediate an increase in permeability to larger macromolecules across the endothelial barrier, suggesting more severe damage. The direct effects of cell-free hemoglobin (CFH, 0–1 mg/ml) on HUVEC macromolecular permeability were determined after 18 h (Figure 2). CFH triggered a dose-dependent increase in ^{14}C-inulin transferred from luminal to abluminal chambers in transwell plates compared to cells treated with vehicle (**Panel 2A**). The highest dose of CFH (1 mg/ml), a clinically relevant concentration directly observed in the plasma of septic patients *in vivo*, [4,8] caused a more than two-fold increase in ^{14}C-inulin transfer. We next determined whether decreases in barrier integrity were temporally associated with cell death after CFH challenge. CFH did not cause a dose-dependent change in cell viability after 18 h compared to control cells as measured by trypan blue exclusion (**Panel 2B**). These data suggest that CFH increases endothelial monolayer permeability within 18 h by a mechanism independent of cell death.

Cell-free hemoglobin depletes intracellular ascorbate

Because we have previously shown that intracellular ascorbate is critical for maintenance of endothelial barrier integrity [20,21,22]. we tested whether CFH altered levels of intracellular ascorbate. Confluent HUVECs were challenged with increasing doses of CFH (0–1 mg/ml) for 18 h and intracellular ascorbate was measured by HPLC. CFH triggered significant dose-dependent decreases in intracellular ascorbate levels (Figure 3).

Ascorbate inhibits cell-free hemoglobin-induced permeability increases

To determine whether ascorbate blocks increases in endothelial permeability triggered by CFH, confluent HUVECs grown in ascorbate-free medium were loaded with ascorbate (0–60 μM) for 15 min prior to challenge with CFH (0.3 mg/ml). After 18 h, ascorbate prevented decreases in monolayer resistance caused by CFH (Figure 4A). Similarly, transfer of ^{14}C-inulin was significantly lower in cells pre-treated with ascorbate compared to those untreated (Figure 4B).

DISCUSSION

In this study we show that CFH decreases monolayer integrity, increases macromolecular permeability, and decreases intracellular ascorbate in human endothelial cells (HUVEC). These effects can be reversed by pre-treatment with ascorbate at physiologic concentrations. These results provide a mechanistic link between the observations that CFH causes endothelial dysfunction [23] and that ascorbate (vitamin C) is critical for maintenance of endothelial barrier integrity [12,20,21,22]. The observation that CFH can deplete intracellular ascorbate may have particular relevance to conditions such as sepsis, which are characterized by both increased circulating CFH [4] and ascorbate depletion [14].

Hemoglobin, a potent oxidant once released from the confines of the red blood cell, is a driver of vascular dysfunction in a variety of disease states. For example, in the setting of chronic hemolysis, the vascular effects of CFH on larger muscular arteries are mediated by reductions in nitric oxide bioavailability [5]. In sickle cell disease, the severity of hemolysis with release of CFH is associated with pulmonary hypertension [24,25,26], which is mediated, in part, by nitric oxide depletion by CFH [5]. Similarly, hemodialysis is associated with episodic release of CFH and decreased NO bioavailability leading to vascular dysfunction [27]. While much is known about the effects of CFH on vascular dysfunction in chronic hemolysis, relatively little is know about its acute effects on endothelial permeability. CFH decreases monolayer electrical resistance in dermal microvascular endothelial cells through a MyD88-dependent mechanism [23] and in bovine lung microvascular endothelial cells specifically in response to hemoglobin alpha [28] but no cellular mechanism of these effects was explored. Our data extend these studies to demonstrate that CFH can mediate endothelial permeability through depletion of intracellular ascorbate. Together these studies support that CFH can acutely increase microvascular permeability and identifies a novel mechanism, vitamin C depletion, to explain this effect.

Ascorbate is a critical cofactor for maintenance of a healthy endothelium. Our group has previously shown that depletion of intracellular ascorbate in cultured endothelial cells leads to increased paracellular permeability [21,22]. Ascorbate helps to tighten the endothelial barrier through stabilization of the tubulin cytoskeleton [22]. Despite this understanding of the critical role of ascorbate in endothelial barrier integrity, little is known about the specific factors that cause intracellular ascorbate depletion. Intracellular ascorbate stores are depleted as ascorbate functions as a reducing agent (electron donor) in an oxidative milieu to reduce oxidant stress. In this study, we demonstrate that CFH is one stimulus that can deplete intracellular ascorbate in cultured endothelial cells. CFH also increases endothelial permeability which can be attenuated by treatment with ascorbate, confirming that the mechanism of CFH-induced permeability is through ascorbate depletion. This conclusion is supported by prior data showing that CFH can be reduced by ascorbate [15]. Overall, our findings may have particular relevance to conditions characterized by both ascorbate depletion and liberation of CFH, such as sepsis [4,8,13,14]. In fact, ascorbate treatment has been [29] and is currently being studied (NCT02734147, NCT02106975) in patients with sepsis.

In summary, we show that CFH depletes intracellular ascorbate leading to increased paracellular permeability of endothelial cells. These effects can be attenuated by ascorbate supplementation. These findings may have clinical relevance to conditions associated with both ascorbate depletion and CFH release such as sepsis.

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Highlights

- Cell-free hemoglobin (CFH) increases endothelial barrier permeability
- Endothelial ascorbate is depleted following exposure to CFH
- Treatment of endothelial cells with ascorbate attenuates CFH mediated permeability

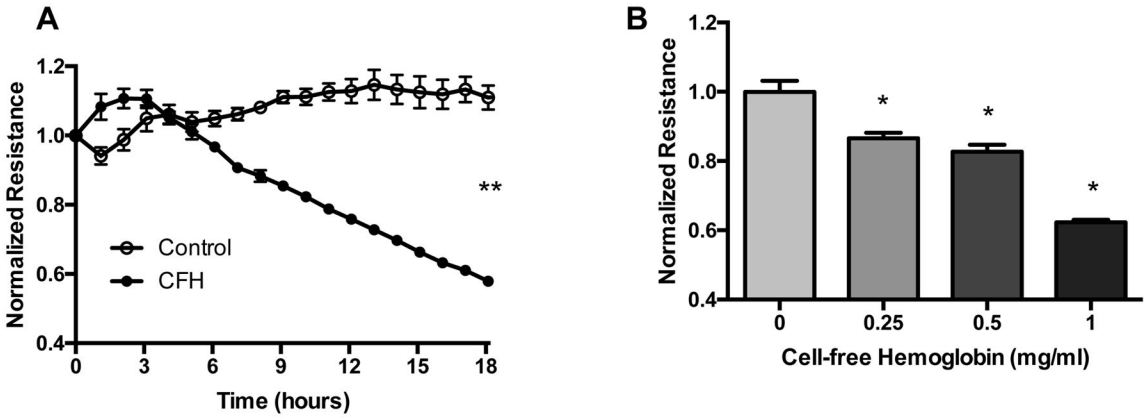

Figure 1. Cell-free hemoglobin decreases electrical resistance across an endothelial cell monolayer

HUVECs were cultured to confluence on ECIS plates and electrical resistance was measured at 4,000 hz over time in response to 1 mg/mL CFH (A). In addition, CFH decreased electrical resistance at 18 hours in a dose-dependent manner (B). **p<0.05 by Mann Whitney U at 18 hours, *p < 0.05 by ANOVA.

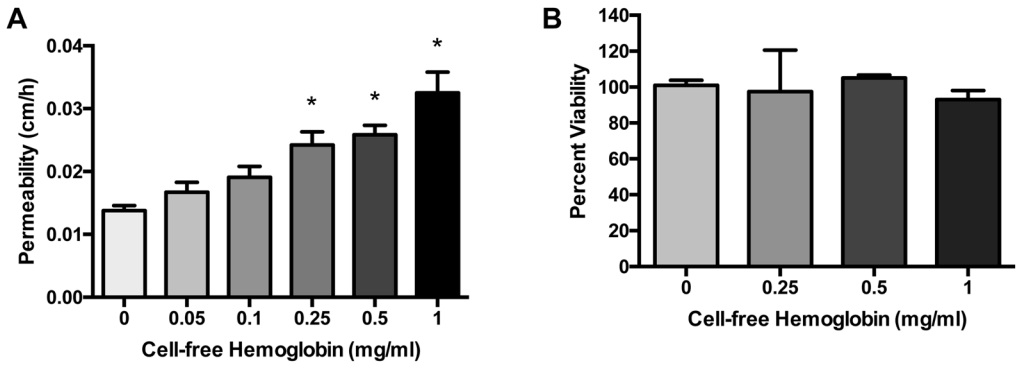

Figure 2. Cell-free hemoglobin increases endothelial macromolecular permeability without altering viability

HUVECs were cultured to confluence on transwell plates and treated with cell-free hemoglobin (CFH, 0–1 mg/ml) for 18 h. CFH increased transfer of ^{14}C-inulin (A) in a dose-dependent manner. Cell viability as measured by Trypan blue exclusion was not affected by CFH treatment for 18 hours (B). *$p < 0.05$ by ANOVA.

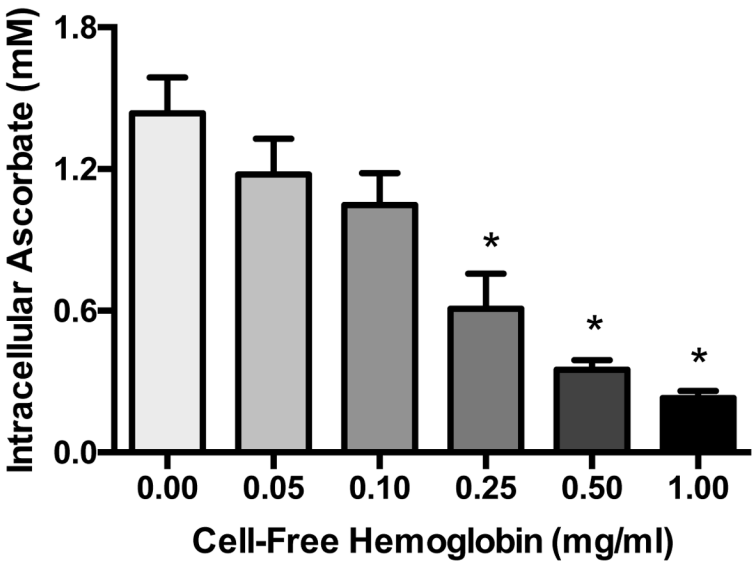

Figure 3. Cell-free hemoglobin depletes intracellular ascorbate

Confluent HUVECs were challenged with CFH (0–1 mg/ml) in antioxidant-free medium for 18 h, then intracellular ascorbate was measured by HPLC. *$p < 0.05$ by ANOVA.

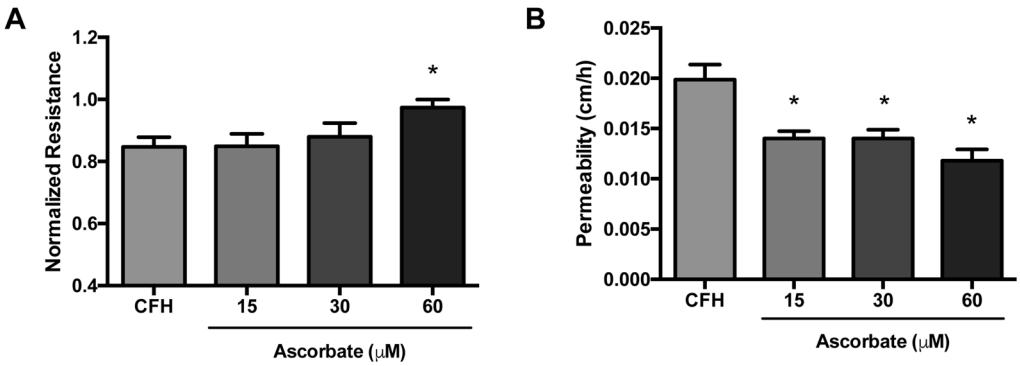

Figure 4. Ascorbate prevents increases in endothelial permeability caused by CFH

HUVECs were cultured to confluence in antioxidant-free medium on transwell plates and loaded with ascorbate (0–60 uM) for 15 min prior to challenge with cell-free hemoglobin (CFH, 0.3 mg/ml) for 18 h. Ascorbate significantly blocked CFH mediated decrease in electrical resistance (A) and the increased transfer of ^{14}C-inulin (B). *$p < 0.05$ by ANOVA.