Biochemical and Biophysical Research Communications xxx (2017) 1–5

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Ascorbic acid attenuates endothelial permeability triggered by cell-free hemoglobin

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ARTICLE INFO

Article history:
Received 2 November 2017
Accepted 8 November 2017
Available online xxx

Keywords:
Cell-free hemoglobin
Endothelial permeability
Ascorbic acid
Oxidative stress

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 h after 1 mg/ml CFH, $p < 0.05$). Pre-treatment of HUVECs with ascorbate attenuated CFH induced permeability.
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.

1. 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

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https://doi.org/10.1016/j.bbrc.2017.11.058
0006-291X/

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.

2. Materials and methods

2.1. 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).

2.2. 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 h.

2.3. Endothelial permeability

2.3.1. 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.

2.3.2. 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.

2.4. 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 × *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].

2.5. 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 (1 mM) to remove extracellular hemoglobin.

2.6. 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 $p < 0.05$.

3. Results

3.1. 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 (Fig. 1A) and dose-dependent (Fig. 1B) decrease in electrical resistance, consistent with disruption of the endothelial barrier by CFH.

3.2. 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 (Fig. 2). CFH triggered a dose-dependent

Please cite this article in press as: J.L. Kuck, et al., Ascorbic acid attenuates endothelial permeability triggered by cell-free hemoglobin, Biochemical and Biophysical Research Communications (2017), https://doi.org/10.1016/j.bbrc.2017.11.058