Blood Cells, Molecules and Diseases 54 (2015) 302–306

Contents lists available at ScienceDirect

Blood Cells, Molecules and Diseases

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

Haptoglobin attenuates hemoglobin-induced heme oxygenase-1 in renal proximal tubule cells and kidneys of a mouse model of sickle cell disease

Narendranath Reddy Chintagari [a], Julia Nguyen [b], John D. Belcher [b], Gregory M. Vercellotti [b], Abdu I. Alayash [a,*]

[a] Laboratory of Biochemistry and Vascular Biology, Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, MD 20993, USA
[b] University of Minnesota, Department of Medicine, Vascular Biology Center, Division of Hematology, Oncology and Transplantation, Minneapolis, MN 55455, USA

ARTICLE INFO

Article history:
Submitted 5 December 2014
Accepted 11 December 2014
Available online 22 December 2014

(Communicated by M. Narla, DSc, 11 December 2014)

Keywords:
Sickle cell disease
Kidney damage
Haptoglobin
Hemeoxygenase-1
Kidney proximal tubules

ABSTRACT

Sickle cell disease (SCD), a hereditary hemolytic disorder is characterized by chronic hemolysis, oxidative stress, vaso-occlusion and end-organ damage. Hemolysis releases toxic cell-free hemoglobin (Hb) into circulation. Under physiologic conditions, plasma Hb binds to haptoglobin (Hp) and forms Hb–Hp dimers. The dimers bind to CD163 receptors on macrophages for further internalization and degradation. However, in SCD patients plasma Hp is depleted and free Hb is cleared primarily by proximal tubules of kidneys. Excess free Hb in plasma predisposes patients to renal damage. We hypothesized that administration of exogenous Hp reduces Hb-mediated renal damage. To test this hypothesis, human renal proximal tubular cells (HK-2) were exposed to HbA (50 μM heme) for 24 h. HbA increased the expression of heme oxygenase-1 (HO-1), an enzyme which degrades heme, reduces heme-mediated oxidative toxicity, and confers cytoprotection. Similarly, infusion of HbA (32 μM heme/kg) induced HO-1 expression in kidneys of SCD mice. Immunohistochemistry confirmed the increased HO-1 expression in the proximal tubules of the kidney. Exogenous Hp attenuated the HbA-induced HO-1 expression in vitro and in SCD mice. Our results suggest that Hb-mediated oxidative toxicity may contribute to renal damage in SCD and that Hp treatment reduces heme/iron toxicity in the kidneys following hemolysis.

Published by Elsevier Inc.

Introduction

SCD is a hereditary hemolytic disorder characterized by recurring episodes of painful vaso-occlusive crises and endothelial dysfunction [5]. SCD patients express a mutation in the β-subunit of hemoglobin S (HbS) that promotes polymerization of HbS and the sickling of red blood cells (RBCs) under conditions of low oxygen. The constant sickling and unsickling cycles result in RBC lysis in the microvasculature and the release of acellular HbS [15]. Hp, an endogenous Hb scavenger protein avidly binds to αβ dimers of Hb and forms a highly stable Hb–Hp complex. Binding of Hb to Hp prevents the release of free heme and filtration of Hb by the kidneys. Plasma hemopexin (Hpx) has high affinity for free heme that might be released from metHb [6,30]. In sickle cell disease plasma Hp and Hpx levels are low due to chronic hemolysis [25]. The Hb–Hp complex binds to CD163 receptors expressed on the macrophages of the spleen, liver, bone marrow and kidneys. The Hb–Hp complex is endocytosed and processed intracellularly. Within macrophages, HO-1 mediates the degradation of heme into ferrous iron, carbon monoxide and biliverdin [18]. The iron is safely sequestered as ferric iron by ferritin while biliverdin undergoes further degradation to bilirubin.

Under normal physiological conditions, low levels of Hp–free Hb and heme/iron are metabolized by the kidney via increased expression of HO-1 and H-ferritin [18]. Excessive hemolysis in SCD patients may overwhelm endogenous plasma Hp and other scavenging mechanisms and heme degradation pathways. Acellular Hb is a highly reactive protein which undergoes oxidation to pro-inflammatory methemoglobin and ferryl hemoglobin [26,31]. Moreover, the oxidized Hb species readily lose heme, a highly reactive molecule [3]. Acellular Hb is primarily cleared by the proximal tubules of the kidney via megalin and cubulin receptors [16]. Thus the kidneys of SCD patients are highly susceptible not only to Hb-induced toxicity but also to the deleterious effects of highly reactive heme. Excess amounts of Hb and its degradation products such as heme/iron are implicated in the pathogenesis of SCD [28,33]. The renal manifestations of SCD patients include hematuria, tubular abnormalities, microalbuminuria and sometimes chronic kidney disease [27,28].

Understanding the mechanisms of Hb-induced toxicity may unravel new therapeutic avenues against hemolytic diseases in general and SCD in particular. For example, our recent study revealed that Toll-like receptor (TLR4) antagonists inhibit vaso-occlusion in a model of SCD [6]. Similarly, overexpression of HO-1 reduced hypoxia-reoxygenation induced stasis [7]. Endogenous Hb/heme scavenging proteins are increasingly being investigated for their roles in ameliorating Hb/heme-induced toxicities [30]. Hp reduced acellular Hb-induced renal damage in multiple animal models predominantly by promoting Hb clearance and metabolism

* Corresponding author at: Laboratory of Biochemistry and Vascular Biology, Center for Biologics Evaluation and Research, Bldg. 52/72, Room 4106, Food and Drug Administration, 10903 New Hampshire Avenue, Silver Spring, MD 20993, USA.
E-mail address: abdu.alayash@fda.hhs.gov (A.I. Alayash).

http://dx.doi.org/10.1016/j.bcmd.2014.12.001
1079-9796/Published by Elsevier Inc.

[2,4,8]. Moreover, recent in vitro and in vivo experiments indicated that Hp shields Hb from peroxidative modifications and consequent tissue damage [9]. We hypothesized that Hp may ameliorate Hb-induced toxicity by reducing heme overload in kidney by modulating HO-1 expression as part of a well-developed anti-inflammatory response.

Materials and methods

Isolation of stroma free hemoglobin

Stroma-free human adult Hb (HbA) used for in vitro studies was isolated from whole blood as reported earlier [32]. The isolated Hb was further purified on Superdex 200 column to remove catalase. A spectral analysis was performed to ascertain the quality and the oxidation state of Hb solutions prior to using them in the experiments. Stroma-free human HbA used for in vivo studies was a generous gift from Sangart, Inc. (San Diego, CA).

Haptoglobin solutions

Highly purified Hp solutions were a kind gift from Bio Products Laboratory (BPL, Hertfordshire, UK). The isolation and fractionation of this protein from human plasma were done as previously reported [24]. Typical size-exclusion HPLC separation profiles of Hp samples used in this study revealed the following molecular weight distribution: 60% with 2 αβ (dimer, Hp1–1), 21% with 3 αβ (trimer, mostly Hp1–2), and 19% larger forms (polymer, mostly Hp2–2) [23].

Exposure of kidney proximal tubular cells to hemoglobin

Human kidney proximal tubular cells (HK-2) were purchased from ATCC (Manassas, VA). The cells were cultured in keratinocyte serum-free medium (supplemented with 0.05 mg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor). The media was changed every 48 h (h). The HK-2 cells were exposed to Hb (50 μM; expressed in heme equivalents) and Hp (50 μM; molecular weight 64 kDa) for 24 h. For Hb–Hp experiments, Hp was added separately at equimolar ratio to Hb (50 μM). Following the 24 h incubation, cells were washed with ice-cold Hank's basal salt solution and nuclear and cytosolic protein extracts were isolated exactly as described by the manufacturer (Affymetrix, Santa Clara, CA). Protein concentrations were determined using a Bradford assay (Bio-Rad, Hercules, CA).

Transgenic sickle cell mice

All animal experiments were approved by the University of Minnesota's Institutional Animal Care and Use Committee. We utilized male and female NY1DD transgenic sickle mice in our experiments. The mice were used at 8–12 months of age and the body weights ranged from 20–30 g. They were housed in specific-pathogen-free cages on a 12 h light/dark cycle at 21 °C. All animals were monitored daily including weekends and holidays for health problems, food and water levels and cage conditions. The NY1DD mice are on C57BL/6 genetic background. The NY1DD mice are homozygous for deletion of the mouse β^{major} globin and express a human α and β^S globin transgene. NY1DD mice have no anemia but express a mild disease phenotype. The RBC half-life in these mice is 7 days [14].

Mouse experimental treatments

For studying the effect of Hb, SCD mice were infused with a single bolus of Hb (3.2 μmol heme/kg) or Hp (3.2 μmol/kg). The Hb–Hp complexes (in equimolar ratio) were also infused (0.012 ml/g) as a single bolus. The kidneys were harvested 4 h after infusion, snap frozen in liquid nitrogen and stored at −85 °C until used for analysis or placed into buffered formalin for immunohistochemistry analysis.

RNA isolation, cDNA synthesis and real-time PCR

RNA isolation, cDNA synthesis and real-time PCR (RT-PCR) were carried as previously reported [22]. Briefly, RT-PCR was performed using 100 ng of cDNA on an Applied Biosystems (ABI) TaqMan Gene Expression Assay system and TaqMan Fast Universal PCR Master Mix according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Fluorescence detection was acquired using Applied Biosystems Model 7900HT and the measurements were analyzed through ABI's Version 2.3 Sequence Detection Systems (SDS) software. The ABI "inventoried" mouse specific TaqMan® Gene Expression Assay gene probes were used for studying gene expression. The gene expression was based on actual cycle threshold (dC_t) values. The data were normalized to GAPDH expression. The fold change was based on the log (2) difference of dC_t values between treatment groups [$-\log 2(ddC_t)$].

Western blotting

Proteins were separated via gel electrophoresis using precast 4–20% Tris–glycine mini-gels (Bio-Rad, Hercules, CA). Proteins were transferred onto nitrocellulose membranes as described previously [12]. The membranes were blocked in Tris-buffered saline (pH 7.4) containing 0.01% Tween-20 and 5% skim milk. They were then incubated with rabbit polyclonal antibody to HO-1 (Abcam, Cambridge, MA). Proteins were visualized using enhanced chemiluminiscence system (GE Healthcare Bio-Sciences, Pittsburg, PA). Equal loading was confirmed by re-probing the blots with a rabbit polyclonal antibody to β-actin (Sigma-Aldrich, St. Louis, MO).

Immunohistochemistry

Immunohistochemistry was performed as previously reported [11]. The formalin-fixed tissues were de-paraffinized and subjected to

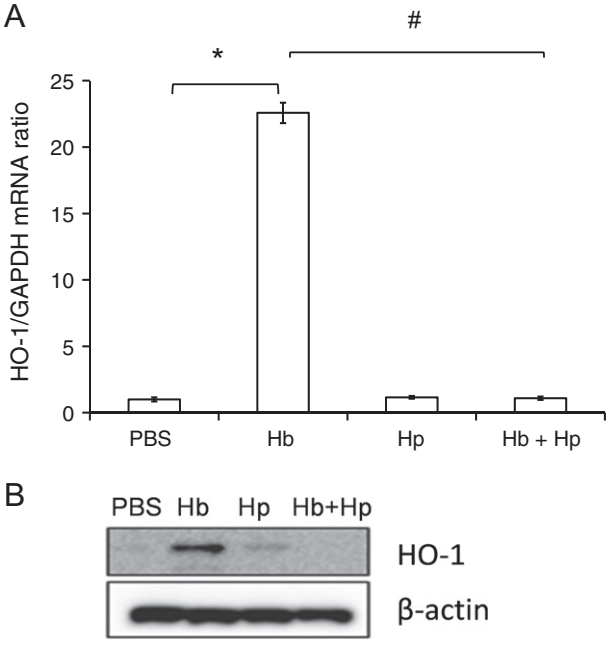

Fig. 1. Haptoglobin attenuates Hb-induced HO-1-expression in human proximal tubule cells: human proximal tubule cells (HK-2) were exposed to Hb [50 μM (heme)], Hp (50 μM) and Hb–Hp (in equimolar ratio; added separately) for 4 and 24 h under normoxic conditions. The cells were washed and immediately lysed for total RNA and protein extractions. The gene and protein expression of HO-1 were monitored by RT-PCR and Western blot respectively. Shown are A) gene expressions of HO-1 following exposure for 4 h. The data were normalized against GADPH and B) HO-1 protein expression following exposure for 24 h. $^*p < 0.05$ vs PBS; $^\#p < 0.05$ vs Hb.