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

Narendranath Reddy. Chintagari[1], **Julia Nguyen**[2], **John D. Belcher**[2], **Gregory M. Vercellotti**[2], and **Abdu I. Alayash**[1]

[1]Laboratory of Biochemistry and Vascular Biology, Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, Maryland, 20993, USA

[2]University of Minnesota, Department of Medicine, Vascular Biology Center, Division of Hematology, Oncology and Transplantation, Minneapolis, Minnesota, 55455, USA.

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 hours. 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.

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-

Correspondence to: Abdu I. Alayash, Ph.D., D.Sc. 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, Maryland-20993. Phone: 240/402-9350 abdu.alayash@fda.hhs.gov.

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 ferrylhemoglobin [26;32]. Moreover, the oxidized Hb species readily lose heme, a highly reactive molecule [2]. 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;34]. 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 [31]. Hp reduced acellular Hb-induced renal damage in multiple animal models predominantly by promoting Hb clearance and metabolism [1;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 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 [33]. 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 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 BioProducts 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 hours (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 hour 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 μmols heme/kg) or Hp (3.2 μmols/kg). The Hb-Hp complexes (in equimolar ratio) were also infused (0.012 ml/g) as a single bolus. The kidneys were harvested 4 hours 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 a Applied

Biosystems (ABI) TaqMan Gene Expression Assays system and TaqMan Fast Universal PCR Master Mix according to 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 Assays 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 chemiluminiscense 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 antigen retrieval. The tissues were permeabilized and incubated with mouse monoclonal antibody to HO-1 (Abcam, Cambridge, MA) at a dilution of 1:250 overnight. The tissues were washed and incubated with secondary antibodies conjugated to horseradish peroxidase for 1 h at room temperature. The localization of protein was visualized using Vectastain ABC systems exactly as per the instructions of the manufacturer (Vector Labs, Burlingame, CA). Nuclei were visualized by counterstaining with hemotoxylin.

RESULTS AND DISCUSSION

Excess free Hb is primarily cleared by the proximal tubules of the kidney. Thus, they are highly prone to Hb-induced toxicity. We used a kidney proximal tubule cell line (HK-2) to investigate their responses following exposure to Hb. HK-2 cells were exposed to 50 μM Hb and 50 μM Hp for 4 or 24 h. For studying the role of Hp in Hb-induced injury, Hb and Hp were added (in equimolar ratio) separately to the cells. Hb induced HO-1 mRNA within 4 h whereas Hp completely attenuated Hb-induced HO-1 mRNA expression (Fig. 1A). We did not observe upregulation of HO-1 protein expression within 4 h (data not shown). However, Hb induced the upregulation of HO-1 protein following exposure to Hb for 24 h. Hp attenuated the Hb-induced HO-1 protein expression (Fig 1B).

We next investigated the *in vivo* kidney responses following infusion of Hb into NY1DD sickle mice. The mice were infused *via* the tail vein with a single bolus of Hb (32 μmols heme/kg) with and without equimolar amounts of Hp or PBS. Hb induced a robust

expression of HO-1 mRNA at 4 h compared to PBS-treated mice, indicating the early induction of HO-1 gene transcription by Hb (Fig. 2A). Hp attenuated Hb-induced HO-1 mRNA expression in SCD mice when compared to Hb alone (Fig. 2A). Hb also induced expression of HO-1 protein in kidney of SCD mice. Hp attenuated the Hb-induced HO-1 protein expression in NY1DD sickle mice 4 h after Hb infusion (Fig. 2B). We also examined the immunolocalization of HO-1 in the kidney of SCD mice following Hb infusion. Immunohistochemistry indicated an increased expression of HO-1 in the proximal tubules of the kidney. Hp attenuated the Hb-induced HO-1 expression (Fig. 2C).

Hp binds to Hb and prevents the clearance of Hb dimers by the kidney. However, the endogenous Hp and other clearance mechanisms are overwhelmed and depleted in hemolytic diseases such as SCD. We set out to show that exogenous Hp if complexed with Hb might ameliorate Hb-induced oxidative toxicity. [2]. Indeed our data show that free uncomplexed Hb induces the expression HO-1 in renal proximal cells *in vitro* and in the proximal tubules of the kidneys *in vivo* in SCD mice. Complexing Hb with Hp and/or administrating Hp attenuated the Hb-induced HO-1expression in kidney tubule cells *in vitro* and *in vivo*. Induction of HO-1 in the kidneys in the absence of Hp could be due to a cascade of oxidative reactions resulting in the loss of heme [2]. .

Acellular Hb is a highly reactive protein which undergoes spontaneous (autooxidation) and/or chemically (hydrogen peroxide)–induced oxidation to higher oxidation states such as metHb and ferrylHb in a pseudoenzymatic fashion that result in oxidative and damaging changes to the protein itself and other biological molecules [3]. Moreover, the oxidized Hb species readily loose heme, a highly reactive metabolite [10]. The potential source of heme in our cell culture model is metHb which is known to lose heme more readily that ferrous Hb. The *in vivo* sources of metHb could be the result of autooxidation of Hb and/or its reaction with NO, which also results in Hb oxidation [29]. We have recently shown that metHb but not cyanometHb (where heme group is blocked with cyanide) induced vaso-occlusion in a SCD mice model [6]. Moreover, HbS undergoes oxidation at a faster rate when compared to the HbA [21]. It has been shown recently that sickle cell erythrocytes, unlike normal ones, possess unique enzymatic mechanisms for reactive oxygen (ROS) production mediated by NADPH oxidases that can be stimulated by plasma signaling factors [17]. In another study, ferryl HbS was shown to interfere with actin remodeling in malarial-infected erythrocytes which confirms the unique oxidative milieu within sickle cell erythrocytes that provides a steady supply of oxidants [13]. We have obtained data recently to show that ferrylHbS persists longer in solutions than ferrylHbA and results in greater oxidative toxicity and mitochondrial dysfunction (unpublished data). Thus, the increased oxidation of HbS predisposes SCD patients to heme overload and likely contributes to the complex pathophysiology of this disease [19]

We have in recent years accumulated considerable experimental data defining the precise mode of action of Hp under oxidative stress conditions. Our data describe a unique protection that Hp provides when it is in complex with Hb. Unlike other chaperon proteins, the Hb-Hp complex allows Hb to consume oxidants such as peroxide by allowing direct excess to the heme active site of Hb. However, Hp short-circuits the emerging radicals including the ferryl protein radicals. Remarkably in spite of the unhindered

pseudoperoxidase activity, no heme is lost from Hb-Hp complex for a long time [20;24]. Our *in vitro* and *in vivo* data clearly support this mechanism which demonstrated that Hp not only attenuated Hb-induced HO-1 expression in HK-2 cells and proximal tubule cells of the kidney but also reduced renal damage following administration of acellular Hb [4;8]. Hp not only controls Hb oxidative side changes but also prevents it from losing the highly reactive and pro-inflammatory heme group resulting in down-regulation of HO-1.

CONCLUSIONS

The results indicate that Hp abrogates Hb-induced HO-1 expression in proximal renal tubule cells not only *in vitro* but also *in vivo*. The results also indicate the possibility of modulating Hb clearance by administration of exogenous Hp to prevent kidney damage in SCD patients.

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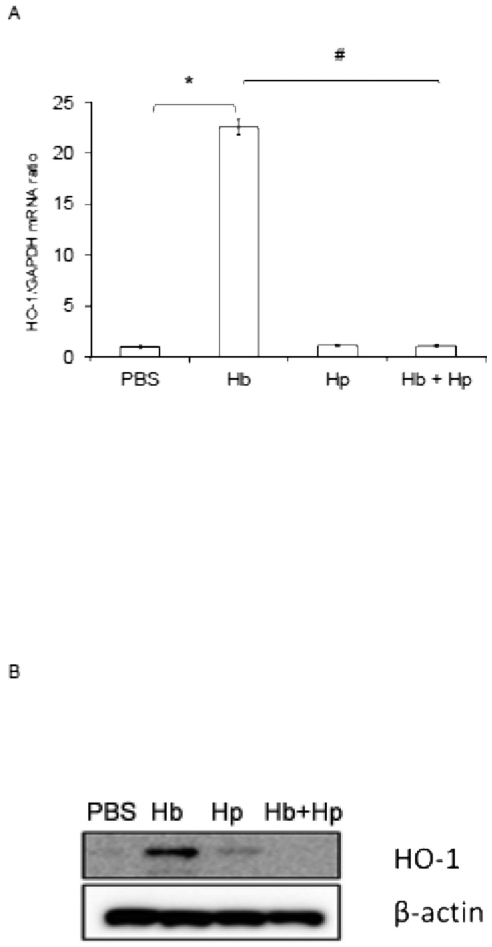

Fig. 1. Haptoglobin attenuates Hb-induced HO-1-expression in human proximal tubule cells

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 hrs under normoxic conditions. The cells were washed and immediately lysed for total RNA and protein extraction. The gene and protein expression of HO-1 was monitored by RT- PCR and Western blot respectively. Shown are A) gene expressions of HO-1 following exposure for 4 hrs. The data were normalized against GADPH and B) HO-1 protein expression following exposure for 24 hrs. *$p<0.05$ vs PBS; #$p<0.05$ vs Hb.

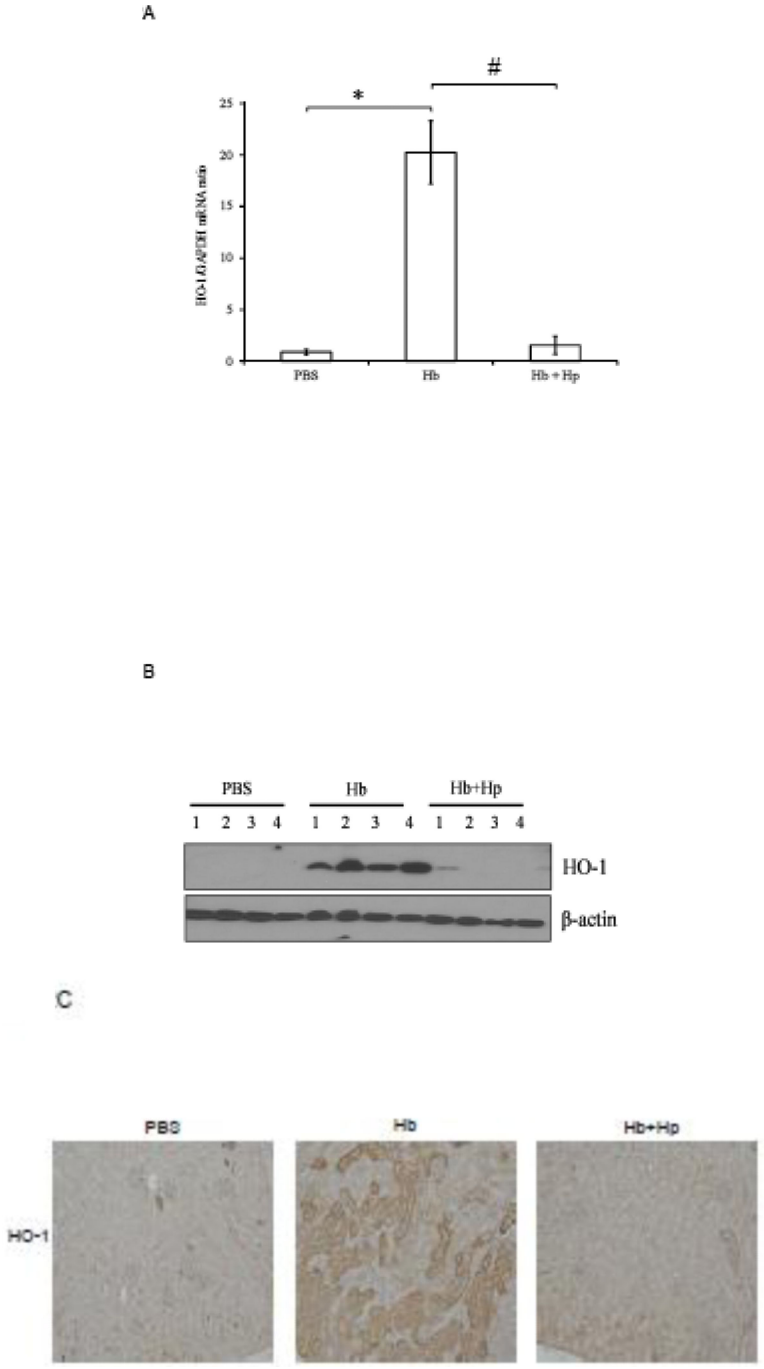

Fig. 2. Haptoglobin blocks HO-1 protein expression in kidney of SCD mouse model

NY1DD sickle mice (n=4/treatment) were infused with saline, Hb, Hp or Hb-Hp complex. Four hours later, the kidneys were isolated and analyzed for the expression of HO-1 by RT-PCR and Western blotting. **(A)** HO-1 mRNA expression. The data were normalized to GADPH mRNA expression and **(B)** western blot images of HO-1 protein expression. The blots were re-probed against β-actin to confirm equal loading. The numbers represent individual animals. The immunolocalization of HO-1 protein in kidneys of NY1DD sickle mice was studied by immunohistochemistry. **(C)** Representative images of HO-1 expression

in mice infused with PBS, Hb or Hb+Hp. The expression of HO-1 was predominantly evident in the proximal tubules of the cortex. *$p<0.05$ vs PBS; $^{\#}p<0.05$ vs Hb . Scale bar: 100 μm.