Haptoglobin buffers lipopolysaccharides to safeguard against aberrant NFκB activation

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Abstract

Haptoglobin, an abundant serum protein, is the major detoxifier of hemoglobin. In addition, haptoglobin modulates the inflammatory response *via* NFκB. However, in some studies haptoglobin represses and in others it activates NFκB signaling. Here, we show that these conflicting results are explainable by the ability of haptoglobin to bind and buffer lipopolysaccharides. While haptoglobin preparations from human serum induced NFκB-dependent transcription, we found that haptoglobin itself was dispensable. The effect was rather caused by lipopolysaccharides associated with haptoglobin. These were apparently enriched during purification. We show that haptoglobin binds lipopolysaccharides from different bacterial species with low micromolar affinities. This will result in competition with toll-like receptor 4, the lipopolysaccharide receptor, which we demonstrate to be sufficient for induction of NFκB-dependent transcription by haptoglobin. Given its abundance in human serum, haptoglobin constitutes a buffer for serum-borne lipopolysaccharides, shielding them from toll-like receptor 4 to safeguard against aberrant inflammatory reactions caused by small lipopolysaccharide fluctuations. Concordantly, NFκB activation in primary macrophages by purified haptoglobin was delayed relative to stimulation with pure lipopolysaccharide. Our findings warrant evaluation of therapeutic benefits of haptoglobin for non-hemolytic conditions as well as re-evaluation of purification strategies. Finally, they allow to disentangle the mechanistic basis of immunosuppression by oncofetal haptoglobin.

Introduction

Haptoglobin (HP) is an acute phase protein [1] that detoxifies free hemoglobin (HB) [2]. On one hand, HP represses lipopolysaccharide (LPS)-induced TLR4 (toll-like receptor 4)-NFkB signalling [3, 4]; on the other hand, it was reported to activate TLR4-NFκB signalling ([5, 6] and this study). Here, we show that the conflicting functions of HP in TLR4-NFκB signalling are explainable by HP's ability to bind and buffer LPSs.

Methods

Cells and reagents

Buffy coats from healthy female donors were from the University Clinic Marburg. MDMs (monocyte-derived macrophages) were differentiated as described [7]. Mixed-type HP was from Sigma and USBio. Ultrapure LPSs were from Invivogen. Proteinase K was from Bioline. Amicon filters were from Merck-Millipore. hTLR4 (Addgene 13086), pcDNA3-CD14 (13645), and pFlag-CMV1-hMD2 (13028) were gifts from Ruslan Medzhi-

tov and Doug Golenbock. pGL4.32[luc2P/NFκB-RE/Hygro] was from Promega. Antibodies were from Novus (α-HP JM10-79), Santa Cruz (α-IκBα sc-371), and Sigma (α–β-actin AC-15). Hemoglobin ELISA was from Bethyl (E88-134).

Expression analyses

RT-qPCR and immunoblots were performed as described [7]. Primer sequences:

IL1B: `TGAAAGCTCTCCACCTCCAGGGACA GAGGCCCAAGGCCACAGGTATTTTG`

IL8: `AGCTCTGTGTGAAGGTGCAGT GATAAATTTGGGGTGGAAAGGT`

RPL27: `AAAGCTGTCATCGTGAAGAAC GCTGTCACTTTGCGGGGGTAG`

RNA was isolated using TRIfast (Peqlab) [7] with pre-isolation *D.melanogaster* S2 spike-in (1:10) and post-isolation ERCC spike-ins (Thermo Fisher). Libraries were prepared with QuantSeq FWD (Lexogen). Sequencing was performed on a NextSeq 550 (Illumina). Data are deposited at GEO (GSE215916).

Size exclusion chromatography and affinity measurements

1 mg HP was run on a Superdex 200 Increase 10/300 (Cytiva) with 500 mM NaCl in PBS to remove LPSs. Microscale thermophoresis was performed as published [8] with freshly RED-NHS–labeled protein.

Results and discussion

HP purified from human serum induced the expression of *IL1B* (>1,000-fold; fig. 1A) in MDMs. By contrast, HB alone did not induce *IL1B* expression (fig. 1A), which together with the fact that the HP preparation contained only spurious amounts of HB (fig. 1B) indicates that HB is dispensable for *IL1B* induction by HP. Transcriptome analysis of HP-treated *versus* control MDMs identified differentially expressed genes that are typical for an NFκB-dependent response (fig. 1C).

The NFκB-type transcriptomic response led to the idea that HP activates NFκB *via* TLR4. To test this, we performed a synthetic complementation assay in HEK293 cells, where only the forced expression of TLR4, CD14, and MD2 led to induction of *IL8* by purified HP (fig. 1D) indicating that TLR4, CD14, and MD2 together are sufficient to confer sensitivity to HP. In line with this idea, an NFκB-responsive reporter was induced by HP only when we complemented TLR4, CD14, and MD2 in HEK293 cells (fig. 1E), demonstrating that purified HP activates NFκB-dependent transcription *via* TLR4.

Since HP binds to TLR4 [6], we reasoned that HP directly activates NFκB-dependent transcription *via*

TLR4. To test this hypothesis, we enzymatically digested HP using proteinase K. Unexpectedly, we found that proteolysis did not abolish NFκB-dependent transcription (fig. 2A). Thus, HP protein is dispensable for the activation of NFκB-dependent transcription *via* TLR4.

The stimulus remained in the supernatant after ultrafiltration with a 10 kDa cutoff (fig. 2A). The canonical agonists of TLR4 are LPSs; we therefore speculated that HP is associated with LPSs. To test this, we checked whether the antagonistic LPS-Rs (from *R.sphaeroides*) competes with the non-protein factor in the HP preparation. LPS-Rs led to reduced *IL8* induction in response to HP (fig. 2B), indicating the presence of TLR4 agonists. Next, we used a sensitive silver stain to detect LPSs in digested HP [9]. The assay revealed distinct high molecular weight bands, which are observed in preparations from different bacteria (especially in clinical isolates [10]) and indicate the presence of long-chain "smooth" LPSs (fig. 2C). Slower migration of discernible leading bands suggests that the lipid A and inner core moieties of the LPSs differ from the *E.coli* reference, which exhibits a ladder of regularly increasing chain lengths. Thus, the detected LPSs largely originate from various bacterial species other than *E.coli*.

Limulus amoebocyte lysate assay detected 2.5–5 ng LPS/µg protein in different HP preparations (not shown)—at 1 mg/ml HP in serum, this translates into >2.5 µg/ml LPS, which is more than two orders of magnitude above reported levels (LPS is present at 1–50 pg/ml in human serum due to intestinal permeability [11]). Since the LPSs are heterogeneous and different from *E.coli* LPS (fig. 2C), contamination of the HP preparations seems unlikely. A more parsimonious explanation for large amount of LPSs in the preparations is given by the fact that LPS-containing complexes are selectively precipitated by ethanol [12]. It is conceivable that the widely used Cohn cold ethanol serum fractionation protocol leads to enrichment of LPS-bound HP.

To further substantiate the claim that co-purified LPSs rather than the HP protein activate NFκB-dependent transcription *via* TLR4, we treated THP-1 cells with HP protein separated from LPS by gel filtration, resulting in poor induction of NFκB target genes (not shown). Microscale thermophoresis indicates K_d values <10 µM for repurified HP and LPSs from three bacterial species (fig. 2D) that represent different lipid A structures. The *S.minnesota* strain R595 produces rough LPS (lacking repeating oligosaccharide units) exclusively; binding of this molecule establishes that HP interacts with the lipid A or inner core moiety (or both).

These results show that commercially available HP contains LPSs such that the addition of these HP preparations to cells leads to the release of LPSs, which in turn activate TLR4. However, activation should be delayed due to limited LPS availability: HP competes with TLR4 for LPS. In line with this notion, we found that IκBα degradation induced by HP was delayed relative to high-dose and low-dose *E.coli* LPS (fig. 2E). IκBα degradation kinetics depend on LPS concentration [13, 14]. Although the deployed HP should contain

an amount of LPSs corresponding to the high-dose *E.coli* LPS condition, IκBα degradation was much slower, indicating that only a comparably low amount of LPSs was released.

Taken together, we show that HP binds LPSs with low micromolar affinities. Our data provide a mechanistic explanation for conflicting observations on the role of HP in NFκB signaling [3–6]. Moreover, they establish HP to function as a buffer for LPSs. This buffering function is relevant because the rate of change of stimulus concentration controls the NFκB response [14–16]. HP dampens variations in LPS concentration by shielding LPS from TLR4, such that larger concentration changes are required to trigger an equivalent response. In agreement with this model, HP reduces LPS-dependent cytokine expression in a dose-dependent manner [3].

HP is used therapeutically for hemolytic conditions [17]. Our findings extend applications to inflammatory states induced by elevated LPS levels in sepsis and chronic conditions such as neurodegeneration [11], psychiatric diseases, inflammatory bowel disease, and metabolic syndrome [18]. Here, increasing LPS buffering capacity by HP may improve clinical outcomes. Importantly, HP isolation procedures should avoid LPS enrichment.

Two main *HP* alleles exist in humans. Allele 2 encodes for a second multimerisation domain, resulting in oligomer formation [1]. HP isoforms may differentially bind LPS (we however observed no overt differences in NFκB activation). The HP precursor expressed from allele 2, zonulin, increases intestinal permeability [19]. Our findings raise the possibility that zonulin binds LPS.

"Oncofetal" HP, which is observed during neoplasia and pregnancy [1], is a much stronger immunosuppressant than normal adult HP [20]; the mechanistic basis of enhanced immunosuppression remains unclear. Our findings suggest that the alternative glycosylation of oncofetal HP [21] potentially alters its affinity towards LPSs. Alternatively, oncofetal HP may regulate inflammation through LPS-independent mechanisms, which if true warrants separation of beneficial from malignant functions of HP.

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Author contributions

L.Z., J.G., H.S., C.B., B.W., and T.A. planned and performed experiments, contributed reagents, and analyzed data. S.-A.F. and O.S. planned experiments. S.-A.F. optimised MST assays and analyzed data. S.A. performed gel filtration. A.N. and T.S. performed high-throughput sequencing. H.-R.C. planned experiments and analyzed data. T.A. drafted the manuscript. T.A. and H.-R.C. wrote the final manuscript.

Disclosure of Conflicts of Interest

None.

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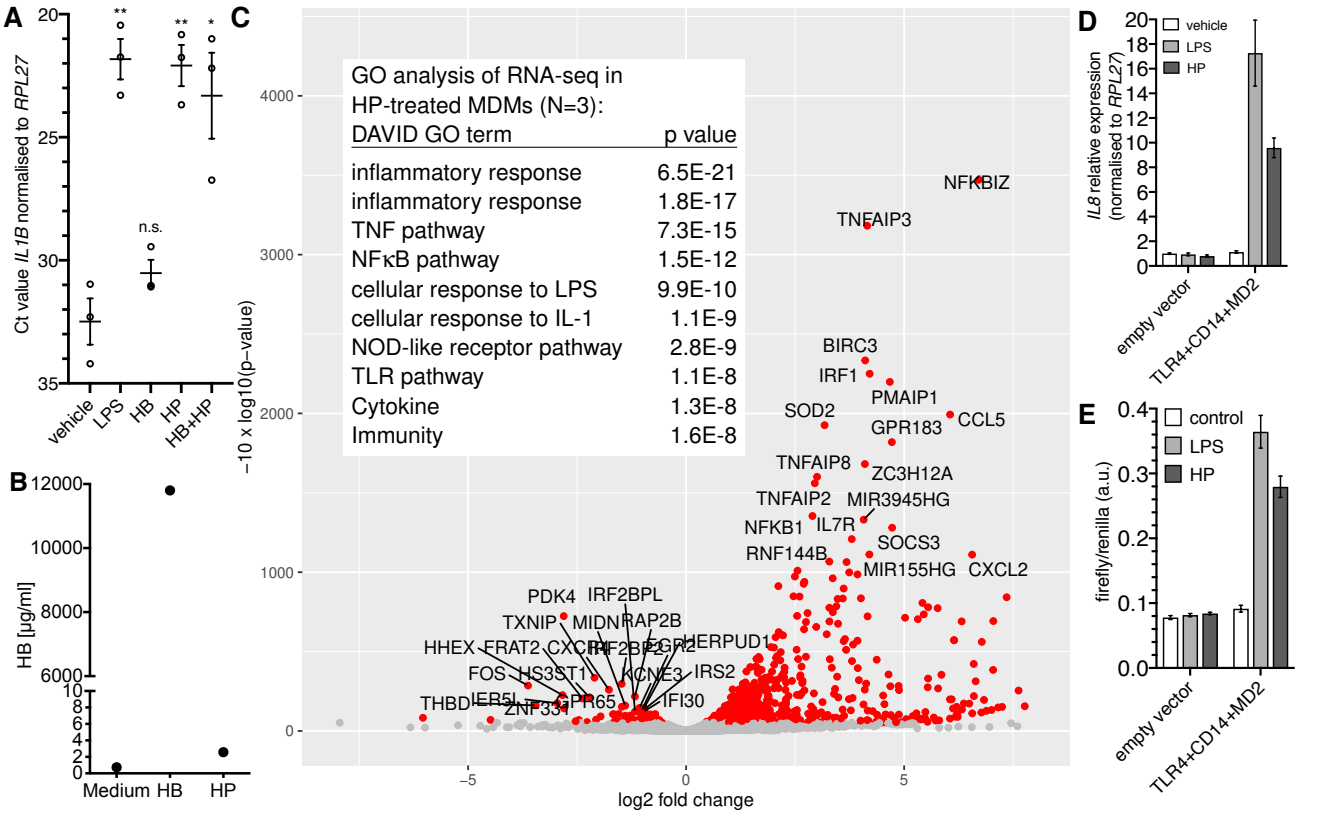

Figure 1: Purified haptoglobin induces NFκB-dependent transcription through TLR4 in the absence of hemoglobin. (**A**) MDMs from blood donors (N=3) were treated with 100 ng/ml *E.coli* LPS or HB (25 µg/ml), HP (25 µg/ml), or both for six hours. Expression of *IL1B* was measured by RT-qPCR. Error bars represent standard deviations. Bonferroni-corrected significance (unpaired t test): **, p<0.01; *, p<0.05; n.s., not significant. (**B**) ELISA analyses of the hemoglobin content of HB and HP. Nominal protein concentration is 10 mg/ml each. (**C**) Volcano plot of RNA-seq data; HP treatment of MDMs from three donors (HP; 25 µg/ml for 4 h *vs.* solvent control). Table inlay: Top ten GO terms assigned by the DAVID database to the top 50 upregulated genes. (**D**) HEK293 cells were transfected as indicated and treated with either solvent, *E.coli* LPS (100 ng/ml), or HP (25 µg/ml) for 6 h. *IL8* expression was monitored by RT-qPCR. This is representative of three independent experiments. (**E**) HEK293 cells were transfected as in A plus an NFκB firefly luciferase reporter plasmid (5×NFκB-luc) and a constitutive *Renilla*-luc reporter and treated as in A. Representative of two independent experiments. D, E: Error bars represent standard deviations from three technical replicates.

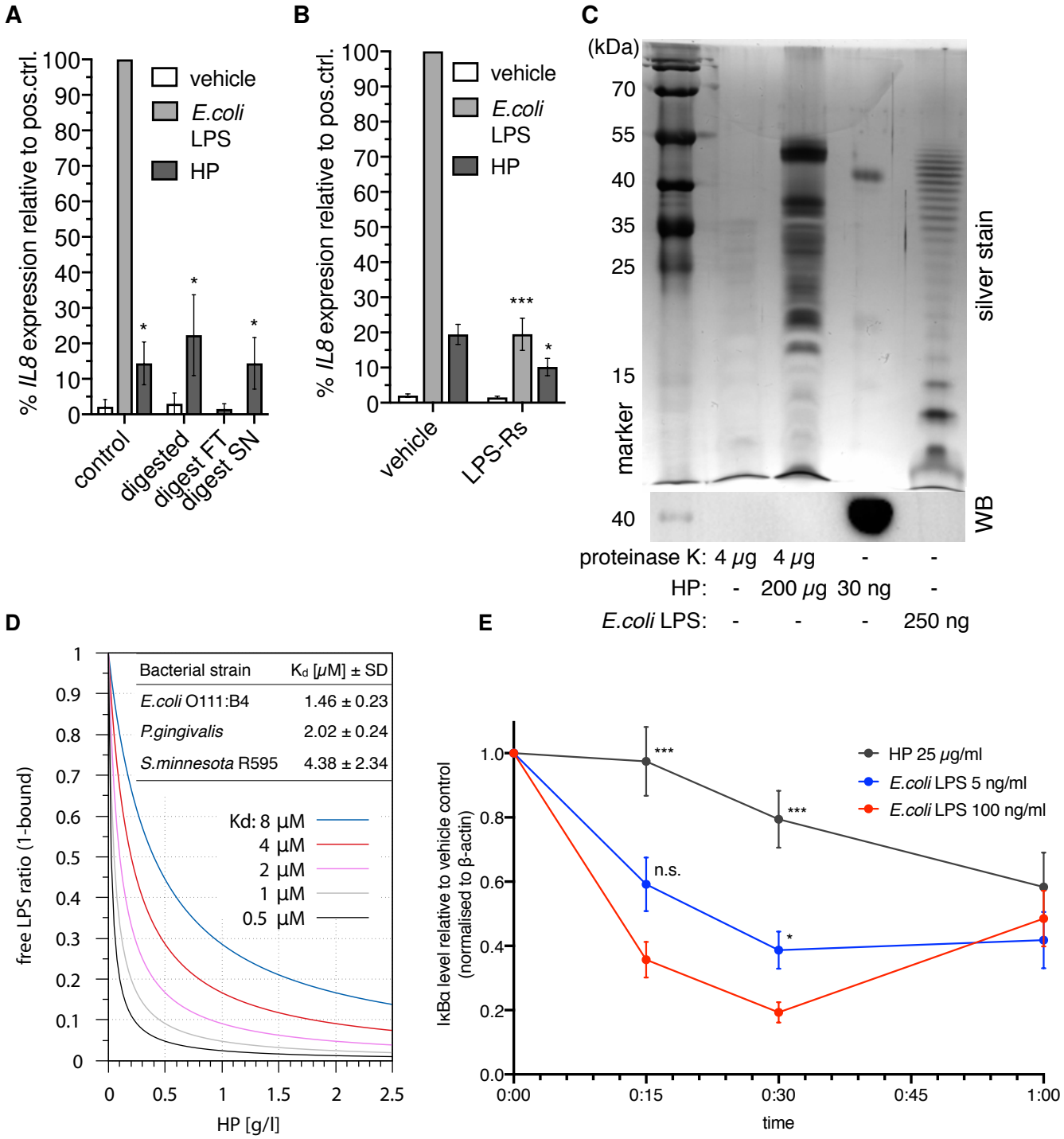

Figure 2: Haptoglobin is associated with lipopolysaccharides and induces delayed NFκB activation. (**A**) HP was treated with proteinase K. An aliquot was ultrafiltrated with a 10 kDa cutoff; FT, flowthrough; SN, supernatant. THP-1 cells were treated for 6 h with these preparations as indicated or with 100 ng/ml *E.coli* O111:B4 LPS, and RT-qPCR of the *IL8* transcript was performed. *, $p<0.05$ relative to the corresponding negative control (unpaired T test). Error bars represent SD. One of two independent experiments is shown. (**B**) Cells were treated as in A with or without LPS-Rs (10 µg/ml). Error bars represent SEM (N=6). ***, $p<0.001$; *, $p<0.05$ relative to the corresponding sample without LPS-Rs (unpaired T test). (**C**) Upper panel: Silver stain of the indicated samples after deoxycholate-urea PAGE under reducing conditions. HP was digested with proteinase K for 2 h at 55 °C or not as indicated. Digestion of the protein was complete as indicated by immunoblotting against the HP β chain (lower panel). (**D**) Calculated ratios of free *vs.* HP-bound LPS at the indicated K_d values. Physiological HP concentrations range from 0.3–2 g/l. At 1 g/l, HP concentration per αβ-subunit is 20 µM. Table inlay: Microscale thermophoresis data obtained with HP repurified using gel filtration and ultrapure LPS preparations from the indicated bacterial strains. Microscale thermophoresis measurements were performed with three HP preparations each. (**E**) IκBα degradation induced by HP shows delayed kinetics relative to pure LPS. MDMs were treated with *E.coli* LPS (100 ng/ml, 5 ng/ml) or HP (25 µg/ml). IκBα levels were monitored by immunoblotting. Chemoluminescence was quantitated with a CCD-based imaging system. IκBα levels were normalised to β-actin. Dots indicate the calculated means (N=7). Error bars denote SEM. Significance relative to high-dose LPS (unpaired T test): ***, $p<0.001$; *, $p<0.05$; n.s., not significant. Significance was Bonferroni-corrected for multiple hypothesis testing.