Endotoxin-neutralizing effects of histidine-rich peptides

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Abstract Inflammatory responses of human peripheral blood monocytes to the Gram-negative endotoxin lipopolysaccharide (LPS) are enhanced by structurally diverse substances, such as anionic polysaccharides or cationic polypeptides. Only a few substances are known to effectively blunt LPS-induced monocyte activation. We now show that synthetic poly-L-histidine (Hn) binds to LPS and abrogates the release of the proinflammatory cytokine interleukin-8 (IL-8) in LPS-stimulated human whole blood. LPS-induced stimulation of monocytes was strictly pH-dependent with only minor amounts of IL-8 secreted in acidic blood. Maximum levels of IL-8 secretion occurred at a strongly basic pH. Hn inhibition of the release of IL-8 from LPS-stimulated monocytes was observed under acidic, neutral and physiological conditions. With increasing alkalosis, the effectiveness of Hn was gradually lost, suggesting that protonated, but not deprotonated, Hn was effective in inhibiting LPS-induced monocyte responses. Histidine-rich protein 2 from the malaria parasite, *Plasmodium falciparum*, inhibited the ability of LPS to evoke an inflammatory response in CD14-transfected THP-1 cells. Further, a short synthetic peptide derived from human histidine- and proline-rich glycoprotein also exhibited LPS-inhibitory effects in CD14 transfectants. Taken together, these observations demonstrate the capacity of histidine-rich peptides, irrespective of their origin, to neutralize LPS-induced proinflammatory host responses.
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Key words: Histidine; Peptide; Monocyte; Endotoxin; *Plasmodium*

1. Introduction

Endotoxins such as bacterial lipopolysaccharides (LPS) activate monocytes and macrophages through a number of LPS recognition molecules, such as CD14 [1] and CD11b/CD18 [2,3], or through class A macrophage scavenger receptors [4]. CD14, for example, is a glycosylphosphatidylinositol (GPI)-anchored protein that responds to subnanomolar concentrations of LPS [1]. The unique sensitivity of CD14 for LPS depends on LPS-binding protein (LBP), a liver-derived high-affinity LPS-binding protein present in human plasma [5]. Plasma-borne LPS is rapidly captured by LBP [5], and the resulting LPS–LBP complex is recognized by cell-associated CD14 [1]. Subsequent association of CD14 with Toll-like receptor 4 [6] triggers a signaling pathway that releases a number of proinflammatory mediators, notably tumor necrosis factor-α (TNFα) [7], a cytokine that plays a pivotal role in the development of septic shock syndrome [8].

Bactericidal permeability-increasing protein (BPI) shares extensive amino acid sequence identity with LBP [5], and, like LBP, binds LPS with high affinity [5]. Interestingly, BPI inhibits LPS-induced cell activation [9]. The opposing effects of the two closely related LPS-binding proteins, LBP and BPI, on LPS activation demonstrate the complexity with which LPS responses are modified.

Previously, we have shown that CAP37 and other arginine-rich cationic polypeptides enhance LPS-induced monocyte activation in human peripheral blood [10]. In addition, we have demonstrated that the enhancement of LPS responses is not a unique property of LPS-binding proteins. Heparin and fucoidan, two polysulfated carbohydrates, for example, increase LPS-induced TNFα production in isolated human monocytes [11,12].

There are relatively few naturally occurring LPS-neutralizing substances, and these mostly consist of cationic polypeptides, such as BPI [9] or CAP18 [13]. In this paper, we show that at physiological pH, poly-L-histidines (Hn) neutralize the effects of LPS, as measured by the suppression of LPS-induced interleukin-8 (IL-8) production in human whole blood. LPS inhibition was also observed with histidine-rich protein 2 (HRP2), derived from the malaria parasite, *Plasmodium falciparum*, and with a synthetic 25-mer peptide, identical to a histidine-rich consensus sequence of human histidine- and proline-rich glycoprotein (HPRG). The data presented here provide evidence that histidine-rich peptides are novel LPS antagonists and set the stage for the exploration of their therapeutic potential.

2. Materials and methods

2.1. Reagents

RPMI 1640 medium, phosphate-buffered saline (PBS), citric acid, *Escherichia coli* 0111:B4 LPS, fluorescein isothiocyanate (FITC)-conjugated LPS (LPS-FITC), Hn, poly-L-arginine (Rn), high-capacity nickel chelate affinity matrix (Ni-CAM® HC Resin), and trypan blue were purchased from Sigma (St. Louis, MO, USA). Sodium carbonate and imidazole were obtained from Merck (Darmstadt, Germany), and recombinant IL-8 was acquired from PharMingen (Becton Dickinson, Franklin Lakes, NJ, USA). Finally, HRP2 was kindly provided by Hans-Peter Beck (Tropical Institute, Basel, Switzerland).

2.2. Whole blood assay

Human whole blood from healthy donors was collected in ethylenediaminetetraacetic acid (EDTA) and acid citrate dextrose vacutainers (Becton Dickinson). Citrated whole blood was immediately diluted with an equal volume of RPMI 1640 medium. Where indicated, 0.3 M citric acid or 0.3 M Na_2CO_3 was added for pH adjust-

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ment. Subsequently, LPS (10 ng/ml), Hn (10 µg/ml), Rn (10 µg/ml), or imidazole (1 mM) were added where indicated. Samples were then incubated for 4 h at 37°C, and supernatants were stored at −20°C. EDTA blood was used to determine the number of peripheral blood monocytes. Briefly, cells were stained for endogenous peroxidase and analyzed by flow cytometry on an ADVIA120 instrument (Bayer Diagnostics, Munich, Germany).

2.3. In vitro binding assay

Fifty microliters of packed nickel chelate affinity matrix were washed in PBS and incubated overnight at room temperature in 1 ml of PBS containing 10 mg of Hn. The matrix was washed in PBS to remove unbound Hn and 100 µl of saline was added, in the presence or absence of 10 µg LPS-FITC, with or without 10 mg Hn. After an additional incubation period of 2 h at room temperature, the matrix was washed in PBS, and bound fluorescence was measured at 485 nm using a FLUOstar SLT microplate fluorescence reader (TECAN Group, Mannedorf, Switzerland).

2.4. Enzyme-linked immunosorbent assay

Supernatants were thawed and the levels of IL-8 or TNFα were determined by sandwich enzyme-linked immunosorbent assay (ELISA). MaxiSorp™ Surface Nunc-Immuno™ Plates (Life Technologies Invitrogen, Basel, Switzerland) were used to maximize sensitivity. Plates were coated overnight at 4°C in 0.1 M carbonate buffer (pH 9.5) using pretitrated antibodies (OptEIA™) from PharMingen (Becton Dickinson). Absorbance at 405 nm was measured using a Titertek Multiskan device from Flow Laboratories (ICN Pharmaceuticals, Costa Mesa, CA, USA).

2.5. Cell culture

Stably transfected THP-1 cell lines expressing GPI-anchored human CD14 [14] were a gift from Jerome Pugin (University Hospital, Geneva, Switzerland) and were cultured as described [15]. CD14 transfectants were incubated at 37°C for 4 h in serum-free AIM-V® medium (Invitrogen, Basel, Switzerland) with or without LPS (10 ng/ml), in the presence or absence of HRP2 (10^{-7} M). CD14 transfectants were resuspended in serum-free AIM-V® medium, containing LPS (10 ng/ml) and increasing concentrations (10^{-9} M, 10^{-7} M, 10^{-6} M, 10^{-5} M, 10^{-4} M) of human HPRG-derived peptide (residues 383–407). After 4 h at 37°C, supernatants were collected and stored at −20°C.

2.6. Peptide synthesis and purification

A 25-mer peptide with the sequence $^+$H$_3$N-GHHPHGHHP-HGHHPHGHHPHGHHPH-COO$^-$, corresponding to residues 383–407 of human HPRG, was synthesized on an Applied Biosystems 433A peptide synthesizer as described [16]. The peptide was then purified by high-performance liquid chromatography (AKTA purifier, Pharmacia Biotech Amersham Biosciences, Otelfingen, Switzerland) on a µRPC C2/C18 column (4.6 mm in diameter and 100 mm in length; Pharmacia Biotech), using a linear solvent gradient (first solvent, 0.1% trifluoroacetic acid (TFA); second solvent, 0.08% TFA in 84% acetonitrile). The product was lyophilized and resolved in 1 M acetic acid. Its purity was demonstrated by acid hydrolysis and amino acid analysis using electrospray mass spectrometry on an API III triple-quadrupole biomolecular mass analyzer (SCIEX Applied Biosystems, Foster City, CA, USA).

2.7. Statistical analysis

Data were analyzed by analysis of variance and Fishers' probable least-squares difference test. P values smaller than 0.05 were considered to be significant.

3. Results

3.1. Synthetic Hn polymers bind to LPS

We first examined whether synthetic Hn polymers bind specifically to LPS. Hn was immobilized on a nickel chelate affinity matrix, and aliquots of Hn-saturated matrix samples were equilibrated with or without LPS-FITC. As shown in Fig. 1, the binding of LPS-FITC to matrix-immobilized Hn was specific, as demonstrated by the complete abrogation of
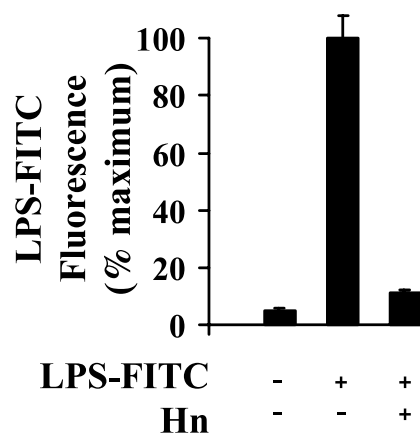

Fig. 1. Binding of LPS-FITC to Hn. Aliquots of nickel chelate affinity matrix (50 µl), saturated with Hn, were incubated with either saline, saline containing LPS-FITC (10 µg) or saline containing a mixture of LPS-FITC (10 µg) and excess free Hn (10 mg). Matrix samples were washed and associated fluorescence measured. Data are expressed as mean value ± 1 S.D., expressed as percentage of maximum detectable fluorescence ($n = 3$).

the binding of LPS-FITC to immobilized Hn after the addition of excess free Hn together with LPS-FITC.

3.2. Hn suppresses LPS-induced production of IL-8 in human whole blood

We next tested the effect of LPS on the production of two proinflammatory cytokines, IL-8 and TNFα, in citrated and RPMI-diluted human whole blood. In a preliminary experiment, we found that in diluted blood samples that were processed immediately after the addition of high concentrations of LPS (1 µg/ml), IL-8 and TNFα levels were undetectable ($n = 5$). However, in diluted whole blood samples that were incubated at 37°C for 4 h after the addition of LPS (1 µg/ml), levels of IL-8 and TNFα were 272 ± 159 and 1403 ± 423 pg/10^5 monocytes, respectively ($n = 5$). Interestingly, incubating diluted blood samples with low (10 ng/ml) or high (1 µg/ml) concentrations of LPS for 4 h at 37°C resulted in the production of similar amounts of IL-8 (10 ng/ml LPS: 210 ± 156 pg IL-8/10^5 monocytes ($n = 5$); 1 µg/ml LPS: 254 ± 200 pg IL-8/10^5 monocytes ($n = 5$); $P > 0.05$). This observation, together with the fact that septic patients typically exhibit low plasma levels of LPS, identifies IL-8 as a suitable marker to study the anti-inflammatory effects of LPS-neutralizing substances.

Next, we examined the effect of Hn on IL-8 production in LPS-stimulated human whole blood (Fig. 2A). The addition of LPS to human whole blood (10 ng/ml) resulted in significant accumulation of extracellular IL-8, whereas treatment with Hn alone (10 µg/ml) did not induce IL-8 secretion. However, when Hn (10 µg/ml) was added together with LPS (10 ng/ml), IL-8 production was suppressed to a level that was barely detectable. The pH of citrated blood samples varied only slightly, ranging from pH 7.0 to pH 7.1.

3.3. Hn-mediated suppression of LPS-induced IL-8 production in whole blood is reversed by imidazole

Having shown that Hn effectively suppresses LPS-induced inflammatory responses (Fig. 2A), we next examined the effect of imidazole on IL-8 production in LPS- and Hn-treated human whole blood (Fig. 2B). In this experiment, it was again

shown that Hn (10 µg/ml) completely suppressed LPS-induced IL-8 production. Imidazole (1 mM) alone did not affect IL-8 production, and it did not significantly affect LPS-evoked IL-8 production in the absence of Hn (10 µg/ml). However, if imidazole (1 mM) was added together with Hn (10 µg/ml), the suppressive effect of Hn on LPS-induced IL-8 production was abolished. There was even a moderate enhancement of LPS-induced IL-8 production (compared with the level of

IL-8 induced by LPS alone). The underlying mechanism for this enhancement is currently unknown. Taken together, these results suggest that the neutralization of LPS-induced increases in IL-8 production by Hn is mediated by the imidazole ring structures that are present within Hn.

3.4. Hn-mediated suppression of LPS-induced IL-8 production in whole blood is reversed by synthetic L-arginine polymers

The observed suppression of LPS-induced IL-8 production by Hn could simply reflect an Hn-associated cytotoxicity. To address this possibility, we used Rn to examine if monocytes were capable of producing IL-8 in response to the addition of Hn. First, we stimulated human whole blood with LPS (10 ng/ml), thereby inducing IL-8 secretion (Fig. 2C). As observed previously, IL-8 could not be detected in blood samples that were incubated with Hn (10 µg/ml) in the presence or absence of LPS (10 ng/ml). However, in the presence of both Hn (10 µg/ml) and Rn (10 µg/ml), LPS re-induced IL-8 to levels observed in samples treated with Rn and LPS alone (Figs. 2C and 3C, pH 7.0). In addition to demonstrating that the effects of Rn on enhancing LPS [10] are dominant over the effects of Hn on suppressing LPS, these data show that Hn (10 µg/ml) is not toxic to blood monocytes.

3.5. LPS-induced production of IL-8 is pH-dependent: protonated but not deprotonated Hn neutralizes LPS responses

The pK_a value of L-histidine is nearly identical to the pH of citrated whole blood. As a result, charged forms of L-histidine side chains predominate in acidic blood. Blood alkalosis, on the other hand, creates predominantly uncharged forms of L-histidine. In order to delineate the possible effects of pH on Hn-mediated LPS suppression, we first examined whether LPS responses, per se, depend on pH. Interestingly, LPS-induced IL-8 production showed a notable pH dependence. At an acidic pH (pH 6.6), levels of secreted IL-8 were barely detectable (Fig. 3A). However, substantial LPS-induced IL-8 release was noted at strongly basic pH levels (Fig. 3A, pH 7.8, pH 8.2). Even in the absence of exogenous LPS, small but reproducible levels of IL-8 release were detected at pH 8.2 (Fig. 3A–C), presumably due to LPS contaminants from the laboratory.

In the presence of Hn (10 µg/ml), LPS-induced IL-8 production was completely abolished at acidic (pH 6.6), neutral (pH 7.0) and physiological (pH 7.4) pH (Fig. 3B). However, the observed Hn-mediated suppression of LPS responses was
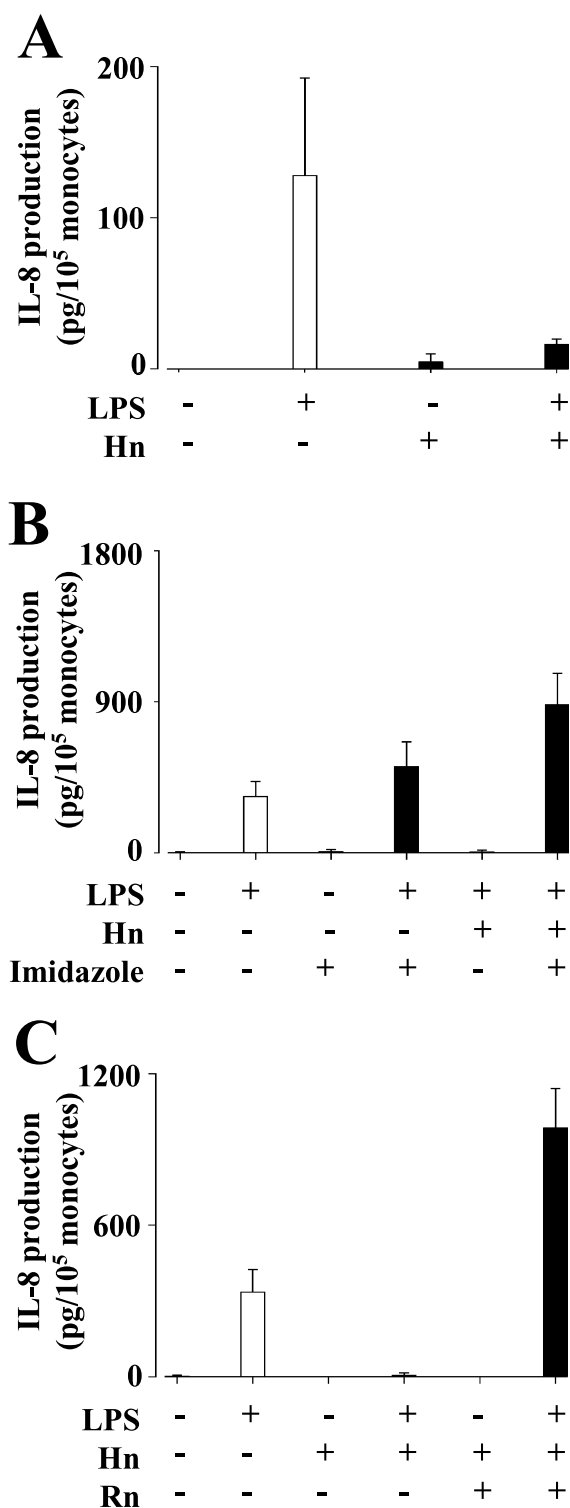
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Fig. 2. A: Effect of Hn on LPS-induced IL-8 production in human whole blood. Citrated whole blood was obtained from four healthy subjects and diluted 1:1 with RPMI 1640 medium. Aliquots of diluted whole blood (pH 7.0–7.1) were treated with or without LPS (10 ng/ml) in the presence or absence of Hn (10 µg/ml). Samples were incubated at 37°C for 4 h, and supernatants were probed for secreted IL-8 by ELISA. Monocyte counts were determined in EDTA blood using automated flow cytometry. Depicted values represent pg amounts of IL-8, released by 10^5 monocytes. B: Effect of imidazole on Hn- and LPS-treated human whole blood. The same experiment as described in A was carried out except that imidazole (1 mg/ml) was added as indicated. C: Effect of Rn on Hn- and LPS-treated human whole blood. The same experiment as described in A was carried out except that Rn (10 µg/ml) was added as indicated. The addition of LPS alone is indicated by white bars. Data are expressed as mean value ± 1 S.D. ($n = 4$).

gradually lost at higher pH levels (Fig. 3B, pH 7.8, pH 8.2, pH 8.6), indicating that cationic, but not neutral forms of Hn were able to neutralize LPS.

Rn, a potent amplifier of LPS responses at neutral and physiological pH [10], enhanced LPS-induced IL-8 production at both acidic and basic pH levels (Fig. 3C). The enhancement was more pronounced at acidic (pH 6.6), neutral (pH 7.0), and physiological (pH 7.4) pH levels (Fig. 3C) and was somewhat less prominent at higher pH levels (Fig. 3C, pH 7.8, pH 8.2, pH 8.6). These observations demonstrate that the diminished capacity of peripheral blood monocytes to mount inflammatory LPS responses in an acidic environment (Fig. 3A, pH 6.6) is not due to cytotoxicity (Fig. 3C, pH 6.6).

Having ruled out the possibility of toxic effects of Hn or acidic pH, we next asked whether variations in pH levels or the presence of poly-L-amino acids (Hn or Rn) could affect IL-8 measurement. To do this, we added recombinant IL-8 (1 ng/ml), Hn (10 μg/ml), and Rn (10 μg/ml) to fetal calf serum diluted 1:1 with RPMI and titrated the samples to the pH values indicated in Fig. 3. Determination of IL-8 levels by ELISA showed that IL-8 detection (the ability to measure IL-8) decreased to 97% at pH 6.6 and 7.8, 95% at pH 8.2, and 90% at pH 8.6, as compared with values measured at neutral or physiological pH. The addition of Hn (10 μg/ml) affected IL-8 detection only slightly (maximum deviation 3%). The addition of Rn decreased IL-8 detection by 4% at acidic, neutral and physiological pH, and by 10% at basic pH.

3.6. Synthetic L-histidine polymers suppress LPS-induced production of IL-8 in CD14-transfected THP-1 cells

In order to test for the ability of naturally occurring histidine-rich proteins or derived peptide sequences to inhibit LPS responses, we established a cell culture system in which LPS activation could be evaluated under serum-free conditions, i.e. in the absence of serum-derived histidine-rich proteins. For this purpose, we used THP-1 cells stably transfected with either the rsv vector alone (rsv) or with an rsv vector containing the sequence for GPI-anchored human CD14 (rsv-CD14) [14]. LPS-induced activation of THP-1 cells was strictly dependent on the presence of the transfected CD14 vector (Fig. 4A). As in human whole blood (Fig. 2A), Hn (10 μg/ml) neutralized proinflammatory LPS (10 ng/ml) effects in CD14 transfectants after a 4 h incubation (Fig. 4A).

To examine whether long-term exposure to Hn was toxic to THP-1 cells, we incubated rsv- and rsv-CD14-transfected THP-1 cells in the presence or absence of Hn (10 μg/ml) for 24 h at 37°C and determined cytotoxicity by counting the number of cells that excluded the cell-impermeable dye, trypan blue. In rsv-transfected cells, cell viability in the presence of Hn was $81 \pm 6\%$ ($n = 5$) and the viability in the absence of Hn was $78 \pm 10\%$ ($n = 5$), where the data are expressed as the number of cells excluding the dye ($P > 0.05$). In rsv-CD14-transfected cells, cell viability was $89 \pm 8\%$ ($n = 5$) in the presence of Hn and $89 \pm 10\%$ ($n = 5$) in the absence of Hn ($P > 0.05$).

Next, rsv-CD14-transfected THP-1 cells were preincubated with both Hn and LPS, and the ability of the cells to produce IL-8 was measured after Hn washout. The cells were preincubated in the presence or absence of Hn (10 μg/ml) and LPS (10 ng/ml) at 37°C for 2 h. After preincubation, cells were washed and fresh medium containing LPS (10 ng/ml) was added. At the end of an additional 2 h incubation period,

the cells were centrifuged at $600 \times g$ for 5 min and IL-8 concentrations were determined in the supernatants. Cells preincubated with saline released 786 ± 26 pg IL-8/10^6 cells, whereas cells preincubated with LPS and Hn released 652 ± 41 pg
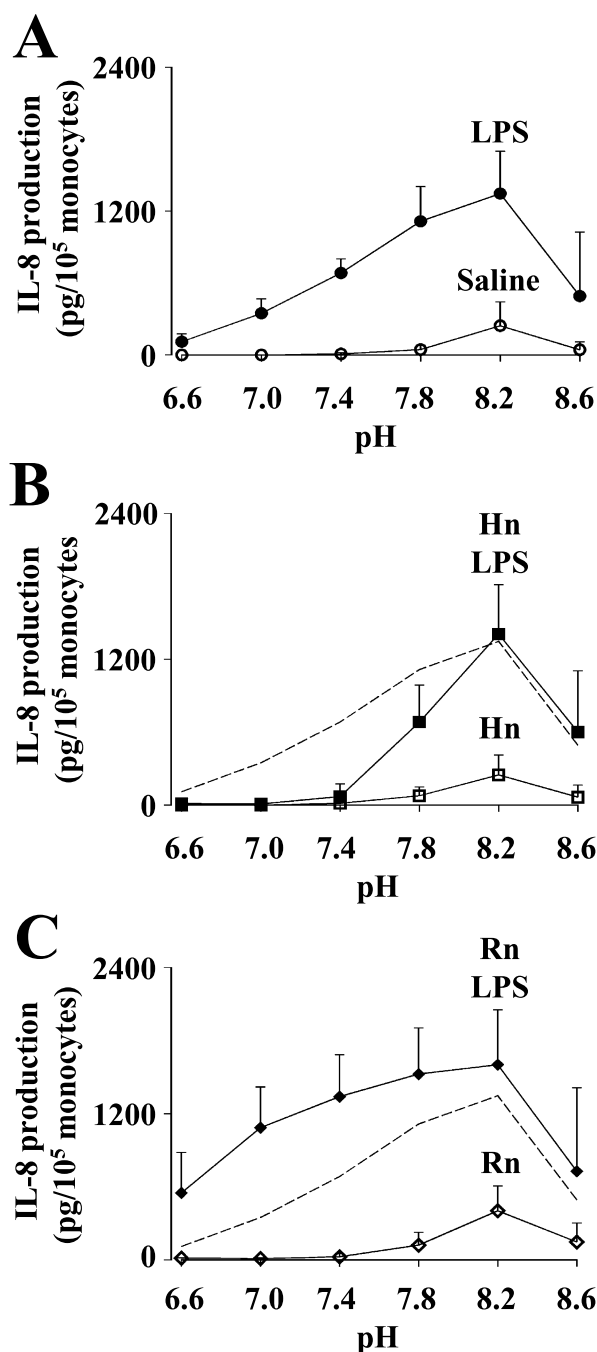

Fig. 3. Effects of varying pH on LPS-induced IL-8 production in human whole blood. Citrated whole blood was obtained from four healthy donors and diluted 1:1 with RPMI 1640 medium. Diluted whole blood (pH 7.0–7.1) was divided into aliquots and the pH was adjusted as indicated, using 0.3 M citric acid and 0.3 M Na_2CO_3. After the addition of saline (open symbols) or 10 ng/ml LPS (closed symbols), samples were divided further and aliquots were treated with (A) saline, (B) 10 μg/ml Hn or (C) 10 μg/ml Rn. Samples were incubated at 37°C for 4 h, and the supernatants were analyzed for secreted IL-8 by ELISA. Monocyte counts were determined by automated flow cytometry. Data are expressed as mean value ± 1 S.D. ($n = 4$). Mean values obtained with LPS treatment alone (A) are indicated by dashed lines in B and C.

IL-8/10^6 cells ($n = 4$ in each group, $P < 0.05$). Because a 24 h incubation with Hn did not affect the viability of transfected THP-1 cells, and because preincubation with Hn and LPS did not abolish LPS-induced IL-8 production 2 h after washout, Hn-mediated cytotoxicity cannot account for the complete lack of IL-8 release in LPS-stimulated CD14$^+$ cells (Fig. 4A).
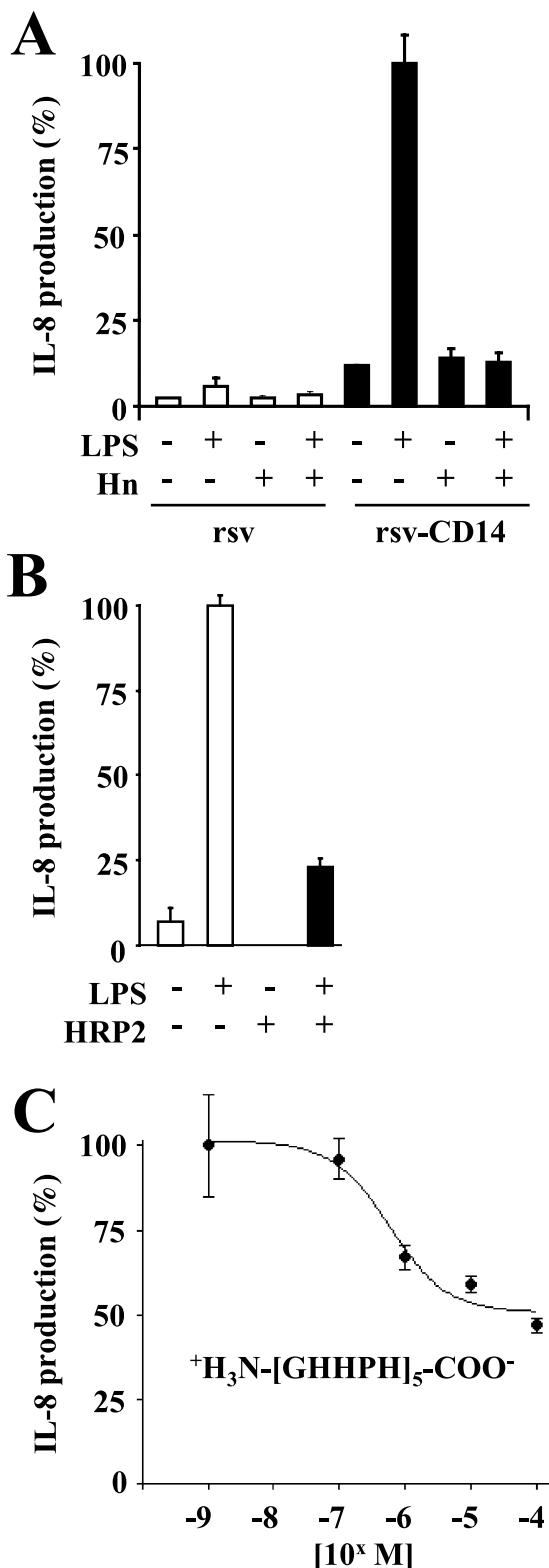
3.7. HRP2, derived from the malaria parasite, P. falciparum, suppresses LPS-induced IL-8 production in CD14-transfected THP-1 cells

CD14-transfected THP-1 cells were resuspended in serum-free medium. A naturally occurring histidine-rich protein, HRP2, which is derived from the malaria parasite, *P. falciparum*, was added in the presence or absence of LPS (10 ng/ml). HPR2 (10^{-7} M) decreased IL-8 production by approximately 80% (Fig. 4B).

3.8. A histidine-rich synthetic peptide, derived from human HPRG, inhibits LPS-induced IL-8 production in CD14-transfected THP-1 cells

A 25-mer peptide, corresponding to residues 383–407 of human HPRG [17], was synthesized and tested for its effects on LPS responses. HPRG$_{383-407}$ dose-dependently inhibited the release of IL-8 in LPS-stimulated CD14-transfected THP-1 cells (Fig. 4C). Taken together, our observations demonstrate that histidine-rich peptides, irrespective of their origin, can neutralize LPS-induced proinflammatory host responses.

4. Discussion

In the present study, we showed that Hn polypeptide chains specifically bind to LPS-FITC. Moreover, Hn chains neutralize the proinflammatory effects of LPS in human whole blood. The use of whole blood preparations requires some form of anticoagulation. The most commonly used anticoagulants are heparin, EDTA, and citrate. Because we were investigating the possible effects of Hn on LPS-induced inflammatory responses in whole blood, heparin was precluded as an anticoagulant because it strongly enhances LPS responses [11]. EDTA, on the other hand, suppresses LPS-induced cytokine production [18].

Histidine is unique among amino acids in that it exists in a partially protonated and partially deprotonated form at physiological pH. Changes in pH are therefore likely to influence the biological effects exerted by histidines. Thus, we performed a systematic analysis of the effects of pH on histidine neutralization of the proinflammatory effect of LPS. We were surprised to find that the LPS-induced release of IL-8 in citrated human whole blood showed a remarkable pH dependence. In acidic blood, the effects of LPS progressively faded. Under conditions of alkalosis, however, LPS-induced IL-8 production increased dramatically. The cause for this pH dependence is currently not known and might be difficult to

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Fig. 4. Effects of HRP2 and HPRG$_{383-407}$ on LPS-induced IL-8 production in CD14-transfected THP-1 cells. A: THP-1 cells were stably transfected with either the empty rsv vector (rsv) or with the rsv vector including the inserted sequence of GPI-anchored human CD14 (rsv-CD14). Cells were incubated in serum-free AIM-V® medium (pH 7.4) for 4 h at 37°C, with or without LPS (10 ng/ml) and in the presence or absence of Hn (10 μg/ml). Supernatants were collected, and secreted IL-8 was determined by ELISA. B: CD14-transfected THP-1 cells were treated with or without LPS (10 ng/ml) in the presence or absence of HRP2 (10^{-7} M), and samples were processed as described in A. C: CD14-transfected THP-1 cells were treated with LPS (10 ng/ml) and increasing concentrations (10^{-9} M, 10^{-7} M, 10^{-6} M, 10^{-5} M, 10^{-4} M) of the HPRG$_{383-407}$ peptide. Samples were processed as described in A. Data are expressed as mean value ± 1 S.D. ($n = 3$).

elucidate, because in diluted whole blood, any number of plasma constituents could contribute to this effect.

Hn specifically bound to LPS-FITC, and Hn-mediated LPS inhibition was associated with positively charged, but not uncharged, forms of L-histidine side chains. Taken together, these findings suggest that the observed LPS-neutralizing effects of Hn were likely to result from electrostatic interactions between histidines and the negatively charged phosphate groups in the lipid A portion of LPS. Phosphate groups are of critical importance for LPS to effectively stimulate target cells. For example, dephosphorylated LPS or LPS from *Rhodospirillum fulvum*, in which the two phosphate residues are replaced by galacturonic acid and heptose, respectively, only induced production of the proinflammatory cytokine IL-6 in human whole blood at high concentrations (above 10 ng/ml) [19].

One of the most histidine-rich proteins identified to date is derived from the bird malaria parasite, *Plasmodium lophurae* [20]. The histidine-rich protein of *P. lophurae*, which has been implicated in erythrocyte invasion [21], contains 74% histidine [22]. The histidines are arranged in stretches of up to nine consecutive residues, sometimes separated by only one or two non-histidine residues. This histidine-rich protein from *P. lophurae* is essentially a naturally occurring variant of the synthetic Hn used in this study. The human malaria parasite, *P. falciparum*, also produces histidine-rich proteins, such as HRP2, which contains 31% histidine [23]. We found that HRP2 effectively neutralizes LPS. Taken together with the fact that charged poly-L-amino acids, such as Hn, are random coils without defined secondary structural features, this finding strongly suggests that a high histidine content alone may be sufficient to blunt LPS responses.

HPRG is an abundant human plasma protein that contains 13% histidine [24]. We tested whether the HPRG-derived consensus sequence, ^+H_3N-(GHHPH)$_5$-COO$^-$, could reduce the proinflammatory effects of LPS. Diminished LPS activation was observed in the presence of this peptide, although a relatively high peptide concentration was required for half-maximal inhibition.

Interestingly, HPRG (average plasma concentration 100 μg/ml) is also known to bind and neutralize heparin [25]. Accordingly, this protein must be first titrated in order for heparin to exert its anticoagulant effects in the bloodstream [26]. Future studies will show whether HPRG buffers LPS as well as heparin, and may thus play an important role in immune defense.

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