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Heparin Binds to Lipopolysaccharide (LPS)-Binding Protein, Facilitates the Transfer of LPS to CD14, and Enhances LPS-Induced Activation of Peripheral Blood Monocytes¹ ✓

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Heparin Binds to Lipopolysaccharide (LPS)-Binding Protein, Facilitates the Transfer of LPS to CD14, and Enhances LPS-Induced Activation of Peripheral Blood Monocytes¹

Michael Heinzlmann^{2*} and Herbert Bosshart^{*†}

Heparin is one of the most effective drugs for preventing and treating thromboembolic complications in surgical patients. Recent evidence suggests that heparin enhances the proinflammatory responses of human peripheral blood monocytes to Gram-negative endotoxin (LPS). We have identified LPS-binding protein (LBP) as a novel heparin-binding plasma protein. The affinity of LBP to heparin was $K_D = 55 \pm 8$ nM, as measured by surface plasmon resonance. Using a fluorescence-based assay, we showed that clinically used heparin preparations significantly enhance the ability of LBP to catalytically disaggregate and transfer LPS to CD14, the LPS receptor. The presence of clinically relevant heparin concentrations in human whole blood increased LPS-induced production of the proinflammatory cytokine IL-8. Fondaparinux, which is identical with the antithrombin III-binding pentasaccharide in heparin, did not bind to LBP or alter LBP function. Thus, this novel anticoagulant drug is a potential candidate for safe administration to patients who have endotoxemia and require anticoagulation. *The Journal of Immunology*, 2005, 174: 2280–2287.

Heparin, a polysulfated glycosaminoglycan, is synthesized in mast cells and stored in secretory granules where it promotes the storage of proteases (1, 2). Although endogenous heparin is not found in the bloodstream, it avidly binds to antithrombin III (AT III)³ and inhibits coagulation when administered exogenously (3). Heparin preparations have been clinically used since the late 1930s (4) to prevent and treat venous thromboembolism (VTE). Similar to the packaging of granule proteases in mast cells, only properly sulfated heparin shows anticoagulant activity (5). In addition to its anticoagulant activity, heparin interferes with the response of peripheral blood monocytes to Gram-negative endotoxin (LPS). Both unfractionated and low-molecular-mass heparin preparations enhance the production of TNF- α in LPS-stimulated cultured human monocytes (6). However, it remains unclear how heparin modifies host responses to bacterial endotoxin.

It is now widely accepted that circulating LPS initially interacts with the soluble LPS recognition molecule, LPS-binding protein (LBP) (7). LPS-LBP complexes are subsequently recognized by the GPI-anchored monocyte differentiation marker CD14 (8). Cellular signaling is transmitted through a coreceptor, the TLR4 (9). The cytoplasmic domain of TLR4 is homologous to that of the IL-1R (10), and activation of TLR4 induces the expression of in-

flammatory cytokines, notably TNF- α (11). The resulting accumulation of TNF- α is thought to be an important factor in the development of septic shock syndrome, which often leads to multiorgan failure and death (12).

Critical molecular interactions in the LPS sensing and signaling pathways have been elucidated (11), and a solid basis for therapeutic intervention in endotoxemic patients is beginning to emerge (13). Many compounds currently under trial to treat septic patients are designed to bind and neutralize LPS, and only a few have molecular targets downstream of the initial LPS-LBP interaction (13). Despite encouraging results from several phase II clinical trials, subsequent larger studies showed that many promising compounds, e.g., recombinant bactericidal permeability-increasing protein (BPI), failed to reduce mortality in septic patients (14). However, it should be emphasized that therapies directed against specific proinflammatory cytokines (e.g., TNF- α and IL-1) are remarkably successful in treating diseases such as rheumatoid arthritis and Crohn's disease (15–17).

Interestingly, LPS binding can result in either activation or inactivation of LPS. Binding of LPS to LBP lowers the threshold at which monocyte responses are triggered in response to subnanomolar concentrations of LPS (8). However, this LPS-enhancing effect is strongly dependent on low serum concentrations of LBP, as Gutschmann et al. (18) have recently shown. These authors demonstrated that higher levels of LBP, such as those found in acute phase serum, neutralized the activation of mononuclear cells by LPS. In contrast, binding of LPS to nanomolar concentrations of BPI does not enhance LPS-induced activation, but instead neutralizes LPS (19). These different effects of LBP and BPI are not easily explained, because LBP and BPI are highly homologous, both bind LPS with high affinity (7, 20), and both are found on the surface of monocytes (21). In contrast, the neutrophil granule protein CAP37, which is not homologous to LBP or BPI, binds LPS (22), binds to and is internalized by monocytes, and enhances LPS-induced activation of monocytes (23). However, none of the diverse structural elements in heparin are found in CAP37 or other LPS-enhancing proteins, suggesting that heparin enhances LPS responses in monocytes by a distinct mechanism.

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³ Abbreviations used in this paper: AT III, antithrombin III; VTE, venous thromboembolism; LBP, LPS-binding protein; BPI, bactericidal permeability-increasing protein; PGN, peptidoglycan; LTA, lipoteichoic acid; ACD, acid citrate dextrose; SPR, surface plasmon resonance.

We now demonstrate that heparin binds to LBP in a strictly sulfate-dependent manner. We provide evidence that this binding results in enhanced LBP catalysis, i.e., enhanced transfer of LBP to CD14. In addition to providing a rational basis for the observation that heparin enhances the effects of LPS, our findings identify LBP as a novel pharmacological target in the modulation of host responses to bacterial endotoxin.

Materials and Methods

Reagents

RPMI 1640 medium, PBS, *Escherichia coli* 0111:B4 LPS, FITC-conjugated LPS (LPS-FITC), heparin-agarose, polymyxin B-agarose, bacterial peptidoglycan (PGN), de-*N,O*-sulfated-*N*-acetylated heparin, and Coomassie brilliant blue R concentrate were purchased from Sigma-Aldrich. BODIPY FL-conjugated LPS was from Molecular Probes. Dextran and dextran sulfate were from Amersham Biosciences. Liquemin (sodium salt of unfractionated heparin) and Lovenox (enoxaparin, a low-molecular-mass heparin preparation) were from F. Hoffmann-La Roche. Atenativ (human AT III and human serum albumin) was from Pharmacia & Upjohn. Atenativ-derived AT III was further purified using a HiTrap Heparin HP affinity column (Amersham Biosciences), according to the manufacturer's recommended protocol. ReoPro was purchased from Eli Lilly and contains human-mouse chimeric Ab fragments, termed c7E3, which recognize the platelet glycoproteins IIb/IIIa as well as CD11b/CD18 complexes on leukocytes (24). Bovine thrombin was from Diagnostec. Arixtra (Fondaparinux sodium) was purchased from Sanofi-Synthelabo. Recombinant human LBP, C-terminally fused to a hexameric histidine tag, and soluble human CD14 were from Biometec. Recombinant murine fractalkine, C-terminally fused to a hexameric histidine tag, was from R&D Systems. *N*-Desulfated-*O*-desulfated and *N,O*-desulfated heparin were kindly provided by Sanofi-Synthelabo. Lipoteichoic acid (LTA) was a gift from T. Hartung (University of Konstanz, Konstanz, Germany).

Whole-blood assay

Human whole blood was obtained from healthy volunteers and collected in anticoagulant Vacutainers containing acid citrate dextrose (ACD) or EDTA (BD Biosciences). The assay was performed essentially as described previously (25). Briefly, citrated whole blood was diluted with an equal volume of RPMI 1640 medium, and LPS, LTA, PGN, heparin, enoxaparin, *N,O*-desulfated-*N*-acetylated heparin, dextran, or dextran sulfate was added as indicated. Samples were incubated for 4 h at 37°C, and the levels of secreted IL-8 were determined by ELISA. Because the number of blood monocytes can vary significantly among healthy individuals, IL-8 levels were normalized to 10⁵ monocytes, as previously described (25). A method that combines staining of endogenous peroxidase with flow cytometric analysis was used to determine the number of monocytes in EDTA-treated blood (ADVIA120; Bayer Diagnostics).

IL-8 measurements

Diluted whole blood samples were spun at 400 × *g* for 5 min, and supernatants were stored at -20°C. Samples were thawed, and the concentrations of IL-8 were determined using a sandwich ELISA. MaxiSorp Surface Nunc-Immuno Plates were purchased from Invitrogen Life Technologies and coated with pretitrated Ab solutions (OptEIA) from BD Pharmingen in 0.1 M carbonate buffer (pH 9.5) overnight at 4°C. Absorbance at 405 nm was measured with a Titertek Multiskan device from Flow Laboratories (ICN Pharmaceuticals).

Heparin binding assay

Heparin-agarose and polymyxin B-agarose were washed in PBS and 50 μl of packed beads were incubated with 10 μg of LPS-FITC at room temperature for 2 h. Beads were washed extensively in PBS and fluorescence levels were determined using a microplate fluorescence reader (FLUOstar SLT; TECAN). To determine whether heparin bound to recombinant or purified native proteins, 1–5 μg of the indicated proteins in 30 μl of PBS was added to 50 μl of washed heparin-agarose beads, and the mixture was incubated at room temperature for 2 h, in the presence or absence of 5 mg/ml heparin, enoxaparin, fondaparinux, *N*-desulfated-*O*-desulfated, *N,O*-desulfated, *N,O*-desulfated-*N*-acetylated heparin, sulfated dextran, or dextran. Unless otherwise indicated, LPS was added at 10 μg/ml. Supernatants and washed agarose beads were boiled in SDS-PAGE sample buffer and run on 10% polyacrylamide gels, as described previously (26). Protein bands were visualized by Coomassie staining, followed by destaining in 40% methanol and 10% acetic acid.

Surface plasmon resonance (SPR)

SPR measures the interaction between an immobilized component referred to as the ligand and a molecule in the mobile phase, the analyte. Changes in surface concentration are proportional to changes in the refractive index on the surface, and this, in turn, results in changes in the SPR signal, plotted as resonance units (RU) with 1000 RU corresponding to a surface concentration of 1 ng/mm² (27). Streptavidin was immobilized on two different surfaces of a CM5 sensor chip at ligand densities of 900 and 1400 RU, respectively, using standard amine coupling chemistry (28). Biotinylated heparin (Celsus Laboratories) was then captured on streptavidin-coated sensor surfaces under saturating conditions at densities of 60 and 115 RU, respectively. LBP was solubilized in PBS (pH 7.4) containing 0.005% Tween 20, and the interaction with immobilized heparin analyzed using a Biacore 2000 instrument (Biacore International). Association and dissociation phases were monitored by injecting a series of LBP dilutions at a flow rate of 20 μl/min. After each injection, heparin-coated surfaces were regenerated with 10 mM NaOH. A deactivated CM5 surface was used to determine unspecific binding and the corresponding resonance unit values were subtracted from resonance unit values obtained using heparin-coated surfaces. A 1:1 binding model assuming Langmuir conditions was applied, and kinetic constants were calculated by nonlinear regression analysis using Biaevaluation software (Biacore, version 4.1).

LPS transfer assay

Disaggregation of BODIPY-LPS and transfer to soluble CD14 was conducted essentially as described (29). The assay uses the fluorescent properties of BODIPY FL, which increases in fluorescence upon transition from an aggregated to a disaggregated state. In aqueous solutions, BODIPY FL-LPS forms micelles, and the associated fluorescence emission represents a fully aggregated state of LPS. The addition of 2% SDS completely solubilizes LPS, and the resulting increase in BODIPY FL fluorescence represents the fully disaggregated state of LPS. The observed increase in BODIPY FL-LPS fluorescence in the presence of LBP and soluble CD14 represents the LBP-catalyzed transfer of monomerized BODIPY FL-LPS to soluble CD14. Briefly, 2 μg of BODIPY FL-LPS, 3 μg of LBP, and 2 μg of soluble CD14 were added to 300 μl of PBS in the presence or absence of 3 mg of heparin (500 IU), 5 mg of enoxaparin (500 IU), and 5 mg of fondaparinux, respectively. After 2 h at 37°C, samples were cooled to 0°C, and carbohydrate-induced quenching of fluorescence was corrected by the addition of heparin, enoxaparin, or fondaparinux to control samples. Relative fluorescence intensities were used to calculate the percentage of disaggregated LPS.

Intravenous heparin injections and blood sampling

Healthy volunteers were injected with 5000 IU of Liquemin, and blood samples were collected in ACD Vacutainers after 1 and 6 h. Before Liquemin injection, an initial blood sample was drawn into ACD Vacutainers to determine LPS-induced IL-8 production in the absence of heparin. Immediately after collection, blood samples were subjected to the whole-blood assay described above. Plasma IL-8 levels were determined after stimulation with 10 ng/ml LPS for 4 h at 37°C. The protocol was approved by the institutional ethics committee, and written informed consent was obtained from all participants.

Statistical analysis

Data were analyzed by ANOVA and Fishers' probable least-squares difference test. Values of *p* < 0.05 were considered to be significant.

Results

Heparin enhances LPS-induced production of IL-8 in human whole blood

We have previously shown that, when isolated human monocytes are treated with heparin or low-molecular-mass heparin, LPS-induced TNF-α production is increased (6). In this study, we tested the effects of heparin on LPS-induced IL-8 production in monocytes in human whole blood. A significant increase in secreted IL-8 accumulation was observed when diluted human whole blood was subjected to short-term stimulation with LPS (Fig. 1A). The addition of standard heparin or the low-molecular-mass heparin, enoxaparin, resulted in 3- to 5-fold amplification of LPS-induced IL-8 production (Fig. 1A).

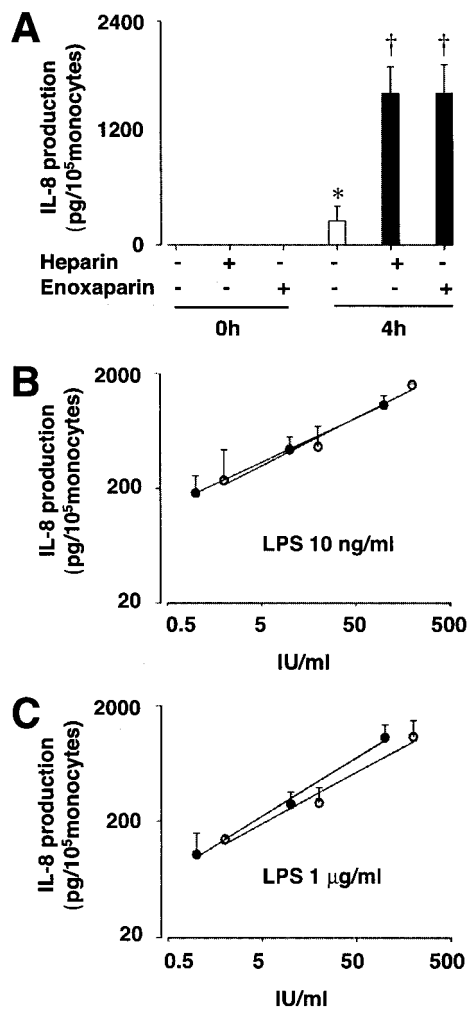


FIGURE 1. Effect of unfractionated and low-molecular-mass heparin on the release of the proinflammatory cytokine IL-8 in LPS-stimulated human whole blood. *A*, Citrated whole blood from healthy donors was diluted 1/1 with RPMI 1640 medium and treated with 10 ng/ml LPS in the presence (■) or absence (□) of 100 IU/ml unfractionated heparin (Heparin) or 100 IU/ml low-molecular-mass heparin (Enoxaparin). Samples were placed on ice immediately (0h) or incubated for 4 h at 37°C (4h). Secreted IL-8 levels were determined in supernatants by ELISA. Data are expressed as mean \pm SD ($n = 5$). * vs †, $p < 0.05$. *B* and *C*, Citrated whole blood from healthy donors was diluted 1/1 with RPMI 1640 medium and treated with 10 ng/ml LPS (*B*) or 1 μ g/ml LPS (*C*). Unfractionated heparin was added at 1, 10, and 100 IU/ml (●) and low-molecular-mass heparin (enoxaparin) was added at 2, 20, and 200 IU/ml (○). Samples were incubated at 37°C for 4 h, and IL-8 levels were determined in supernatants by ELISA. IL-8 production (pg/10⁵ monocytes) is plotted against heparin concentrations (IU/ml) on a double logarithmic scale. Data are expressed as mean \pm SD ($n = 5$). Lines represent linear regression analyses for heparin and enoxaparin.

Heparin and enoxaparin are equally effective in amplifying LPS signals

Treatment of diluted whole blood with either 100 IU/ml heparin or 100 IU/ml enoxaparin (low-molecular-mass heparin) resulted in nearly identical increases in IL-8 production in the presence of 10 ng/ml LPS (Fig. 1A). Thus, we performed a dose-response analysis for heparin and enoxaparin at two different LPS concentrations: 10 ng/ml (Fig. 1B) and 1 μ g/ml (C). Heparin and enoxaparin produced identical dose-response curves at both concentrations of LPS, indicating that 1) 10 ng/ml represents a saturating LPS con-

centration in whole blood, 2) heparin and enoxaparin are equally potent immunomodulators, and 3) LPS contamination of the heparin preparations used can be ruled out as a possible cause of the observed increase in IL-8 production. Because the binding of LPS to CD11b/CD18 (30) activates the same signaling pathway activated when LPS binds to CD14 (31), we tested whether CD11b/CD18 participates in heparin-induced enhancement of LPS. At a concentration of 10 ng/ml LPS, in the presence or absence of 100 IU/ml heparin, the production of IL-8 remained unchanged when CD11b/CD18 was blocked with c7E3 chimeric Ab fragments (data not shown). Because signaling through CD11b/CD18 may require higher concentrations of LPS (32), we performed the same experiment using 1 μ g/ml LPS, with similar results (data not shown). These results indicate that the enhancing effect of heparin on LPS-induced IL-8 production is not mediated through the CD11b/CD18 complex.

Heparin interacts with LBP but not with LPS or soluble CD14

Previous work has shown that isolated human monocytes fail to respond to LPS after CD14 is blocked with specific Abs that prevent the transfer of LPS to CD14, in either the presence or absence

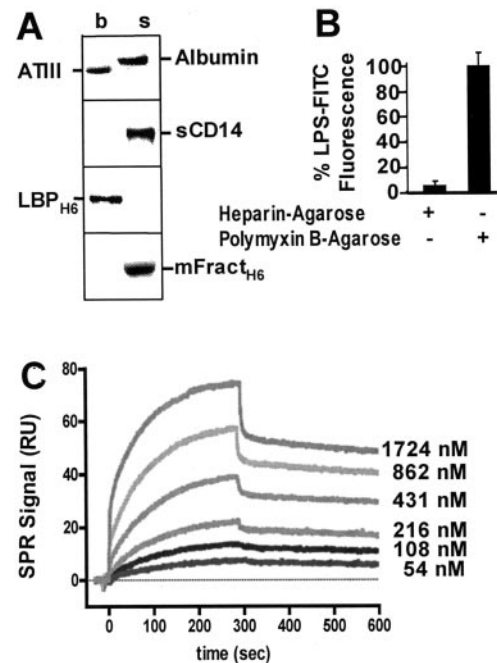


FIGURE 2. Specific binding of heparin to LBP. *A*, Heparin-agarose (50 μ l) was incubated for 2 h at room temperature with 5 μ g of a mixture of human AT III and human serum albumin (Albumin), 2 μ g of recombinant human soluble CD14 (sCD14), 2 μ g of recombinant histidine-tagged human LBP (LBP_{H6}), or 2 μ g of recombinant histidine-tagged murine fractalkine (mFract_{H6}). Agarose beads (b) and supernatants (s) were analyzed separately by SDS-PAGE, and protein bands were visualized by Coomassie staining. *B*, A volume of 50 μ l of packed heparin-agarose or polymyxin B-agarose was incubated with 10 μ g of FITC-conjugated LPS, as indicated. Agarose-bound fluorescence was measured using a spectrofluorometer. Data are expressed as mean \pm SD ($n = 3$). *C*, CM5 sensor chips were coated using streptavidin—at a density of 900 RU, in this example—and biotinylated heparin was captured at a saturating density of 115 RU. Recombinant human LBP was injected at the concentrations (nanomolar) indicated, and SPR units were measured. The ascending curves represent the association of LBP with streptavidin-immobilized biotinylated heparin during LBP injections. The descending curves, generated by switching to running buffer (PBS; pH 7.4) injections, represent the dissociation of LBP from immobilized heparin.

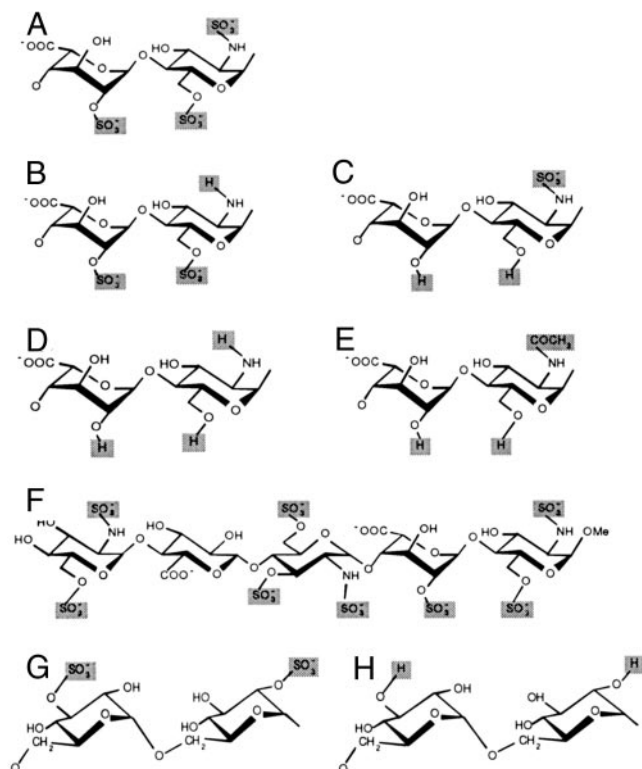


FIGURE 3. Structures of various heparin derivatives. *A*, Clinically used heparin preparations contain a mixture of linear carbohydrate chains. In standard heparin, the length of these chains ranges from 20 to 100 monosaccharides. In enoxaparin, the chain length is reduced to 5–45 monosaccharides. The predominant disaccharide in standard heparin or enoxaparin contains both *N*- and *O*-linked sulfate residues (shaded boxes). *B* and *C*, Partially desulfated heparin derivatives are either *N*-desulfated (*B*) or *O*-desulfated (*C*). *D* and *E*, The completely desulfated heparin derivatives used in this study were *N,O*-desulfated (*D*) and *N,O*-desulfated-*N*-acetylated (*E*). *F*, Chemically synthesized fondaparinux is identical with the AT III-binding pentasaccharide sequence in heparin and enoxaparin. *G*, Dextran sulfate, an *O*-sulfated heparin analog, is branched and consists of ~2000 monosaccharide units. A representative disaccharide is shown. *H*, Dextran is a neutral carbohydrate lacking *O*-linked sulfate residues.

of heparin (6). To test the hypothesis that heparin interferes with LPS activation upstream of the LPS receptor, CD14, we investigated whether heparin could interact with LPS, soluble CD14, or LBP. To do this, agarose-immobilized heparin was incubated with various purified native or recombinant proteins. Binding to heparin was measured by SDS-PAGE analysis of the supernatants and agarose fractions. As shown in Fig. 2*A*, purified human AT III bound specifically to heparin, but purified human serum albumin or recombinant soluble human CD14 did not. Surprisingly, recombinant human LBP was quantitatively bound to heparin-agarose. In contrast, recombinant murine fractalkine, an unrelated protein of similar size and glycosylation, did not interact with heparin. The C-terminal hexameric histidine tag in LBP was excluded as a possible heparin binding site, because an identical tag was present in the C-terminal end of fractalkine. Furthermore, the addition of excess free histidine did not interfere with binding of LBP to heparin (data not shown). To determine the specificity of the interaction of heparin with LPS, we compared the capacities of agarose-immobilized heparin and agarose-polymyxin B to capture FITC-conjugated LPS in solution. Polymyxin B binds LPS with high affinity (33), and the LPS-FITC-associated fluorescence bound to polymyxin B was used as a positive control to quantify the interaction

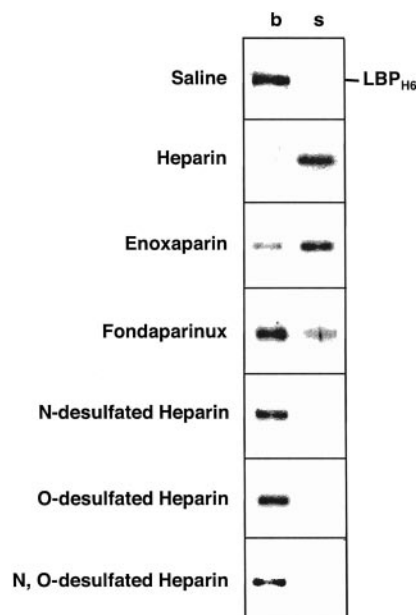


FIGURE 4. Minimal chain length and proper sulfation are both required for heparin binding to LBP. Heparin-agarose (50 μ l) was incubated for 2 h at room temperature with 2 μ g of recombinant histidine-tagged human LBP (LBP_{H6}), in the presence or absence of 625 μ g/ml heparin (100 IU/ml), 1 mg/ml enoxaparin (100 IU/ml), 625 μ g/ml fondaparinux, 625 μ g/ml *N*-desulfated heparin, 625 μ g/ml *O*-desulfated heparin, or 625 μ g/ml *N,O*-desulfated heparin. Agarose beads (*b*) and supernatants (*s*) were analyzed separately by SDS-PAGE and visualized by Coomassie staining. Data shown are representative of results obtained in three separate experiments.

of heparin with LPS. A total of <5% of polymyxin B-associated LPS-FITC was recovered on heparin-agarose beads (Fig. 2*B*). Thus, there was no specific interaction between heparin and LPS-FITC.

Using SPR, we measured the strength of the association between LBP and heparin. Recombinant human LBP showed an association with streptavidin-bound biotinylated heparin in a concentration-dependent manner (Fig. 2*C*, ascending curves). The steady-state distribution between soluble and surface-bound LBP was reached after 4–5 min (Fig. 2*C*). The mobile phase was then changed to running buffer (0.005% Tween 20 in PBS (pH 7.4)). As a result, LBP dissociated from immobilized heparin (Fig. 2*C*, descending curves), rapidly establishing a new steady-state distribution (*C*, 300–600 s). The binding constants obtained in our experiments ($K_D = 55 \pm 8$ nM) were strikingly similar to the one reported for AT III and heparin ($K_D = 57$ nM) (4), indicating that the observed interaction between LBP and heparin was most likely biologically significant.

Binding of heparin to LBP is mediated by sulfate residues and correlates with the enhancement of LPS signals

To characterize the binding between heparin and LBP, heparin-agarose was incubated with LBP in the presence of various chemically modified derivatives of heparin (Fig. 3). As shown in Fig. 4, only soluble heparin and enoxaparin effectively prevented the binding of LBP to immobilized heparin. Fondaparinux, an AT III-binding heparin-derived pentasaccharide, as well as partially or totally desulfated heparin, were all unable to prevent LBP binding to heparin-agarose (Fig. 4). This indicated that both proper sulfation and a minimal chain length are necessary for heparin binding to LBP. The fact that heparin and enoxaparin exhibited both LBP binding (Fig. 4) and LPS enhancement (Fig. 1), suggested that

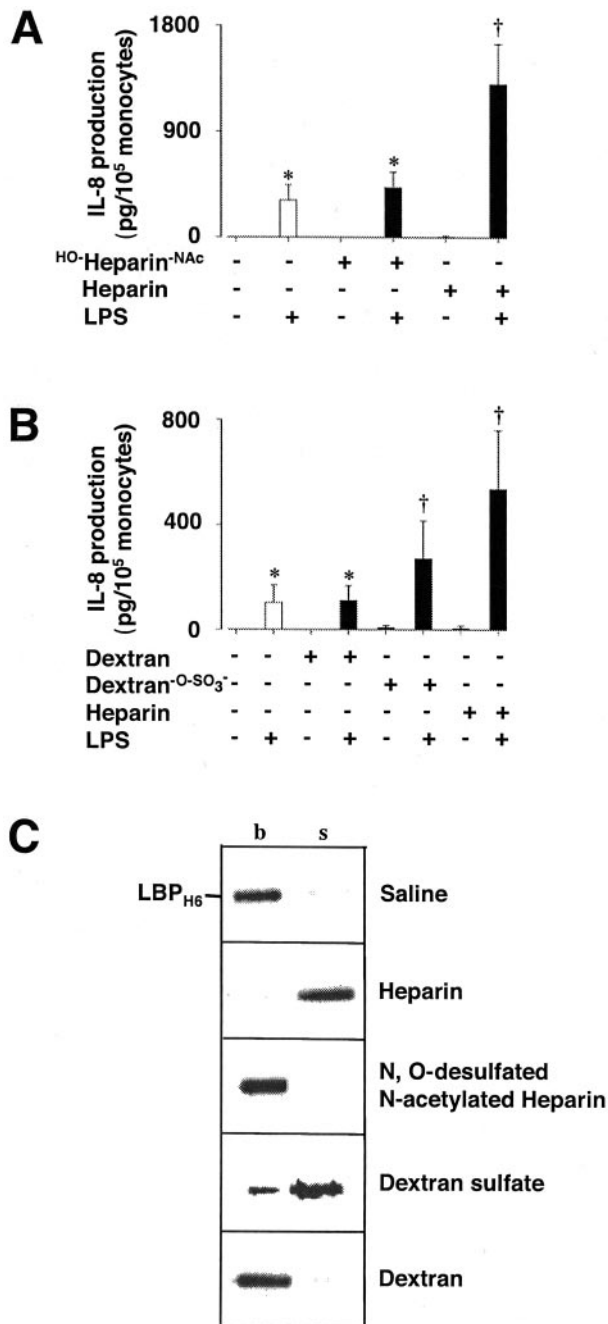


FIGURE 5. Correlation of LPS-induced IL-8 production and LBP binding. *A* and *B*, Citrated whole blood from healthy donors was diluted 1/1 with RPMI 1640 medium and treated for 4 h in the presence or absence of 625 $\mu\text{g/ml}$ (100 IU/ml) unfractionated heparin (Heparin), 625 $\mu\text{g/ml}$ *N,O*-desulfated-*N*-acetylated heparin (HO-Heparin^{NAc}), 1 mg/ml dextran (Dextran), or 1 mg/ml dextran sulfate (Dextran^{O-SO₃⁻) with or without 10 ng/ml LPS, as indicated. IL-8 levels were determined by ELISA. Data are expressed as mean \pm SD ($n = 4$). * vs †, $p < 0.05$. *C*, Heparin-agarose (50 μl) was incubated for 2 h at room temperature with 2 μg of recombinant histidine-tagged human LBP (LBP_{H6}), in the presence or absence of 625 $\mu\text{g/ml}$ heparin (100 IU/ml), 625 $\mu\text{g/ml}$ *N,O*-desulfated-*N*-acetylated heparin, 1 mg/ml sulfated dextran, or 1 mg/ml dextran, as indicated. Agarose beads (b) and supernatants (s) were analyzed separately by SDS-PAGE, and proteins were visualized by Coomassie staining.}

these two properties may be causally linked. Furthermore, fondaparinux had no effect on either LBP binding (Fig. 4) or on enhancement of LPS signals (34). Further analyses showed that

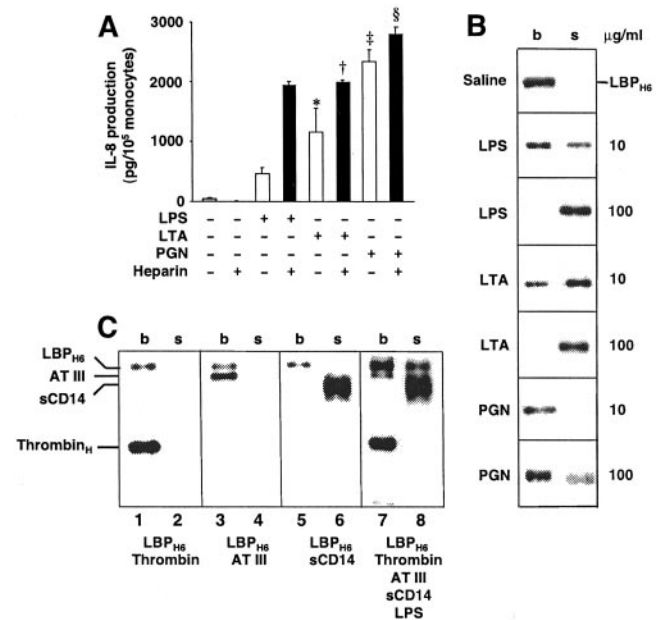


FIGURE 6. *A*, Effects of heparin on TLR2-induced IL-8 production in whole blood. Citrated whole blood from healthy donors was diluted 1/1 with RPMI 1640 medium and treated for 4 h at 37°C with the TLR4 agonist LPS (10 ng/ml) or the TLR2 agonists LTA (10 $\mu\text{g/ml}$) and PGN (10 $\mu\text{g/ml}$), in the presence or absence of 100 IU/ml unfractionated heparin. Secreted IL-8 levels were determined by ELISA. Data are expressed as mean \pm SD ($n = 3$). * vs †, $p < 0.05$; ‡ vs §, $p < 0.05$. *B*, LPS and LTA compete with LBP for binding to heparin. Heparin-agarose (50 μl) was incubated for 2 h at room temperature with 2 μg of recombinant histidine-tagged human LBP (LBP_{H6}), with or without 10 or 100 $\mu\text{g/ml}$ LPS, LTA, or PGN as indicated. Agarose beads (b) and supernatants (s) were analyzed by SDS-PAGE, and protein bands were visualized by Coomassie staining. *C*, LBP binds to ternary complexes of heparin, AT III, and thrombin. Heparin-agarose (50 μl) was incubated for 2 h at room temperature with 2 μg of recombinant histidine-tagged human LBP (LBP_{H6}) and 5 μg of bovine thrombin (lanes 1 and 2, LBP_{H6}, Thrombin), LBP and 5 μg of human AT III (lanes 3 and 4, LBP_{H6}, AT III), LBP and 5 μg of recombinant soluble human CD14 (lanes 5 and 6, LBP_{H6}, sCD14), or 5 μg of LBP, 5 μg of thrombin, 5 μg of AT III, 5 μg of soluble CD14, and 10 $\mu\text{g/ml}$ LPS (lanes 7 and 8, LBP_{H6}, Thrombin, AT III, sCD14, LPS). Agarose beads (b) and supernatants (s) were analyzed by SDS-PAGE, and protein bands were visualized by Coomassie staining. Note that LPS displaces some of the heparin-bound LBP. The mobilities of the individual proteins are indicated on the left.

LBP binding correlated with the ability to amplify LPS responses (Fig. 5). Heparin and dextran sulfate, a structural analog of heparin, both enhanced LPS-induced IL-8 production (Fig. 5, *A* and *B*) and bound to LBP in solution (*C*). In contrast, desulfated heparin and dextran did not bind to LBP (Fig. 5*C*) and had no effect on LPS signals (*A* and *B*). These data strongly support a causal link between LPS enhancement and LBP binding.

Heparin enhances LTA-induced IL-8 production

To better define the role of LBP in the heparin-mediated amplification of LPS signals, we tested whether heparin could also enhance signals induced by Gram-positive LTA. As opposed to LPS, which signals through TLR4, LTA acts via the TLR2 (35). However, like LPS, LTA requires the presence of both LBP and CD14 (36). As shown earlier, heparin enhanced the effect of LPS on IL-8 accumulation by 3- to 5-fold (Figs. 1*A* and 6*A*). Heparin enhanced the effect of LTA (10 $\mu\text{g/ml}$) by 2-fold (Fig. 6*A*). Gram-positive PGN is also a TLR2 agonist that depends on CD14 (37). However,

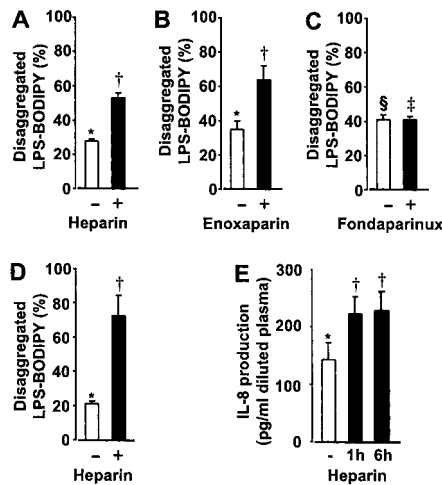


FIGURE 7. A–C, Heparin and enoxaparin, but not fondaparinux, facilitate LBP-dependent disaggregation and transfer of LPS to soluble CD14. BODIPY FL-LPS (2 μ g) was added to 300 μ l of PBS to determine baseline fluorescence of aggregated BODIPY FL-LPS (0% disaggregated). The gain in fluorescence upon addition of 2% SDS was used to define the disaggregated state of BODIPY FL-LPS (100% disaggregated). Test samples contained 2 μ g of BODIPY FL-LPS, 3 μ g of LBP, and 2 μ g of soluble CD14 in 300 μ l of PBS. LPS transfer was measured, in the presence or absence of 3 mg of heparin (500 IU) (A), 5 mg of enoxaparin (500 IU) (B), and 5 mg of fondaparinux (C). Immediately before fluorescence measurements, appropriate amounts of heparin (A), enoxaparin (B), and fondaparinux (C) were added to saline control samples (\square) to correct for fluorescence quenching. * vs \dagger , $p < 0.05$; \S vs \ddagger , $p > 0.05$. D, A volume of 200 μ l of undiluted human serum was mixed with 100 μ l of saline (\square) or 100 μ l of saline containing 3 mg of unfractionated heparin (\blacksquare), and disaggregation of BODIPY FL-LPS (2 μ g) was monitored, as described in A–C. E, Systemic administration of heparin enhanced LPS responses. Four healthy volunteers were injected with 5000 IU of unfractionated heparin, and IL-8 production was assessed ex vivo, in citrated whole blood, diluted 1/1 with RPMI 1640 medium, and stimulated with 10 ng/ml LPS. Preinjection blood samples (\square) were compared with samples drawn 1 and 6 h postinjection. Error bars represent SDs ($n = 4$). * vs \dagger , $p < 0.05$.

it has been reported that the presence of LBP only weakly enhances PGN signals (37). Our results also suggested that the effect of heparin on PGN-induced IL-8 production was much less pronounced than the effects on LPS or LTA (Fig. 6A). Weber et al. (38) recently demonstrated that LBP enhances cell signaling and TNF- α release induced by pneumococcal cell wall products. These authors further showed that LBP specifically binds to pneumococcal cell wall multimers, indicating a novel lipid-independent binding capacity of LBP.

We next examined whether the presence of LPS, LTA, or PGN could interfere with the interaction between heparin and LBP. Biochemical analysis showed that high concentrations of LPS and LTA (100 μ g/ml) prevented LBP from binding to heparin (Fig. 6B). PGN, in contrast, did not significantly compete with heparin binding to LBP, even at high concentrations (Fig. 6B).

LBP binds to ternary complexes of heparin, AT III, and thrombin

We have shown that heparin enhances LPS and LTA responses in whole blood (Figs. 1 and 6). Thus, heparin must interfere with LPS signaling when complexed with the two predominant heparin-binding proteins in human plasma, i.e., AT III (average concentration in plasma, 150 μ g/ml) and thrombin (average concentration in plasma, 100 μ g/ml). Therefore, we next systematically investigated the ability of LBP to bind to agarose-immobilized heparin in

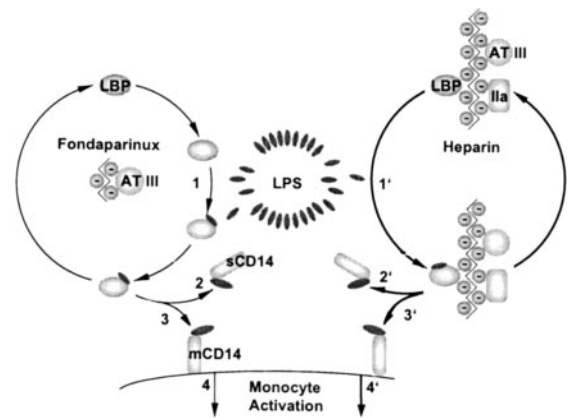


FIGURE 8. A model for the interaction between heparin and LBP. LPS micelles inefficiently activate CD14⁺ monocytes. LBP disaggregates LPS (1) and catalyzes the transfer of LPS monomers to either soluble (2) or membrane-bound CD14 (3). Subsequent activation of monocytes (4) is thought to occur through a physical interaction between membrane-bound CD14-LPS and MD2-TLR4 complexes. We propose that binding of LBP to heparin enhances the catalytic ability of LBP to transfer LPS (1', 2', 3'), thereby resulting in the amplification of LPS signaling (4'). Other heparin-binding plasma proteins such as AT III or thrombin (factor IIa) will also bind to heparin. Plasma concentrations of heparin, LPS, LBP, and soluble CD14 (sCD14), as well as expression levels of membrane-bound CD14 (mCD14) may determine to what extent LPS signals are amplified. Fondaparinux, which is identical with the AT III-binding pentasaccharide sequence in heparin, does not interfere with LPS-induced monocyte activation.

the presence of the predominant LBP- and heparin-binding molecules found in LPS-stimulated whole blood. Heparin-agarose was coincubated with either purified human AT III or purified bovine thrombin and LBP. AT III and thrombin bound to heparin, but this binding did not interfere with the concurrent binding of LBP (Fig. 6C). As shown in Fig. 2A, soluble CD14 did not bind to heparin, nor did its presence interfere with the binding of LBP to heparin (Fig. 6C). When a mixture of LBP, AT III, thrombin, soluble CD14, and LPS was added to heparin-agarose, the bulk of LBP remained bound to heparin (Fig. 6C). To some extent, these experiments mimic the conditions in whole blood, because most other heparin-binding plasma protein are present at much lower concentrations than are AT III or prothrombin (4).

Heparin facilitates the LBP-catalyzed transfer of LPS to soluble CD14

The fact that LPS and LTA both competed with heparin for LBP binding suggested that heparin might interfere with the ability of LBP to bind and/or transfer LPS to CD14. Using a previously described fluorescence-based assay that specifically measures the transfer of LPS to soluble CD14 (29), we evaluated the effect of heparin on LBP-dependent disaggregation and transfer of LPS to soluble CD14. BODIPY FL-LPS dissolved in PBS was used to determine the baseline fluorescence of aggregated BODIPY FL-LPS (0% disaggregated). The gain in fluorescence upon the addition of 2% SDS was used to define the disaggregated state of BODIPY FL-LPS (100% disaggregated). LPS transfer was measured in the presence or absence of heparin (Fig. 7A), enoxaparin (B), or fondaparinux (C), by adding LBP and soluble CD14 to the baseline sample. After incubation for 2 h at 37°C, appropriate amounts of heparin, enoxaparin, and fondaparinux were added to the control samples to correct for carbohydrate-induced fluorescence quenching. Heparin and enoxaparin increased LPS disaggregation by ~2-fold. Fondaparinux, in contrast, had no effect on

LBP-mediated transfer of LPS to soluble CD14. When whole serum was used in place of recombinant LBP and recombinant soluble CD14, heparin enhanced the monomerization of BODIPY FL-LPS by 2- to 3-fold (Fig. 7D), indicating that the addition of exogenous heparin facilitated the transfer of LPS from serum-derived LBP to serum-derived soluble CD14.

To test whether clinically relevant doses of heparin interfered with the LPS-induced release of IL-8, we injected four healthy human volunteers with 5000 IU of heparin, a dose that is commonly used for VTE prophylaxis. Activation of peripheral blood monocytes was measured after *ex vivo* stimulation with 10 ng/ml LPS for 4 h. The experiment showed that LPS-induced production of IL-8 was higher after a bolus injection of 5000 IU of heparin, compared with preinjection levels (Fig. 7E), indicating that heparin, at clinically relevant doses, exhibits proinflammatory properties. The clinical consequences of this result, e.g., for endotoxemic patients who require VTE prophylaxis, are currently unclear and require further investigation (Fig. 8).

Discussion

The pattern recognition receptor, CD14, plays a central role in innate immunity. CD14 is expressed by mature monocytes, and recognizes various bacterial glycolipids such as lipoarabinomannan (39), LTA (36), PGN (37), and muramyl dipeptide (40). Specific binding of polymannuronic acid to CD14 and subsequent signaling (41) has suggested that some glycopolymers might also act as CD14 agonists. Because the chemical structure of polymannuronic acid is similar to that of the anticoagulant drug heparin, we and others have examined the effect of heparin on CD14 signaling. Previously, we showed that both heparin and enoxaparin enhance the release of TNF- α in LPS-stimulated human monocytes in culture (6). Although McBride et al. (42) and Call and Remick (43) reported similar results, Hogasen and Abrahamsen (44) described an inhibitory effect of heparin on the LPS-induced release of proinflammatory cytokines in cultured monocytes. However, this inhibitory effect was observed only under serum-free conditions.

In the present study, we show that heparin enhances LPS responses in human whole blood. The enhancement was similar for unfractionated and low-molecular-mass heparin preparations, suggesting that short heparin molecules with an average chain length of 15 sugar residues are sufficient to mediate this effect. To identify the mechanism by which heparin enhances LPS signals, we determined whether heparin interacts with any of the extracellular molecules known to participate in CD14 signaling, including LPS, LBP, and soluble CD14. A role for extracellular components is consistent with the fact that monocytes no longer respond to LPS after the LPS receptor, CD14, is blocked with an Ab, in either the presence or absence of added heparin (6). We found that heparin binds to LBP but not to LPS or soluble CD14. Because heparin binds to a large number of plasma proteins (4), LBP binding to heparin and heparin-induced enhancement of LPS signaling could be coincidental. However, the fact that heparin, enoxaparin, and the heparin analog dextran sulfate all enhanced LPS signaling and bound to LBP makes this unlikely to be coincidental. The specificity of LBP binding to heparin and heparin-induced enhancement of LPS signaling was further substantiated by the fact that neither desulfated heparin nor dextran bound to LBP or affected LPS signaling. Furthermore, neither LBP binding nor LPS enhancement was observed in response to the synthetic pentasaccharide fondaparinux (34), suggesting that this novel compound could be useful for treatment of endotoxemic or septic patients who require VTE prophylaxis.

LPS, LTA, and PGN all activate monocytes by binding to CD14, but only LPS and LTA exhibit strong LBP dependence (8,

36, 37). Indeed, the heparin-induced enhancement of CD14-mediated signals was most pronounced for LPS and LTA and weaker for PGN, suggesting that LBP plays a role in this process. The observation that LPS and LTA both competed with heparin for LBP binding suggested that heparin might interfere with the ability of LBP to bind and transfer LPS. Heparin and enoxaparin, but not fondaparinux, enhanced this catalytic property of LBP. Our findings show that clinically used heparin preparations 1) enhance LPS responses in human whole blood, 2) bind to LBP, and 3) enhance the transfer of LPS to soluble CD14. We propose that the mechanism by which heparin enhances LPS signaling is analogous to the mechanism by which heparin inhibits coagulation, i.e., through binding and activation of AT III. Several observations support this hypothesis. First, LPS stimulation experiments were conducted with whole blood from healthy volunteers using LPS concentrations between 10 ng/ml and 1 μ g/ml. Second, LBP concentrations in plasma from healthy volunteers range from 2.5 to 4.0 μ g/ml, indicating that LBP was not limiting in our experiments. Finally, LBP exhibits a higher affinity for LPS than CD14 (45). Taken together, these observations suggest that LPS is predominantly bound to LBP and not to CD14 in LPS-stimulated whole blood. Heparin could bind to LBP and lower the affinity of LBP for LPS, resulting in enhanced transfer of LPS to CD14. However, this hypothesis has not yet been directly tested.

Cavaillon et al. (46) described an inhibitory effect of various polysaccharides, including heparin and dextran sulfate, on the binding of LPS to PBMC. Rather than PBMC, we used soluble CD14 as an LPS acceptor, because the affinity of LPS for PBMC includes binding to various surface structures, e.g., CD14 (8), CD11b/CD18 (31), macrophage class A scavenger receptors (47), and, most importantly, the membrane bilipid layer itself (48). The analysis of CD14-dependent binding of LPS to PBMC in the presence of heparin is further complicated by the fact that both heparin and LPS compete for binding to CD11b/CD18 (31, 49). Biochemical analysis using purified LBP, soluble CD14, and heparin, obviates these difficulties. Dziarski et al. (37) reported that, in the absence of LBP, heparin and dextran sulfate have little or no effect on the binding of soluble CD14 to agarose-immobilized LPS. In this study, we show that, in the presence of LBP, heparin and enoxaparin both enhance LPS binding to soluble CD14. Interestingly, enhanced LPS transfer was not observed in response to fondaparinux. This could be clinically relevant, because systemically administered heparin, at doses used in VTE prophylaxis, is sufficient to enhance LPS-induced proinflammatory responses in whole blood. Clearly, further studies will be required to establish whether fondaparinux can be safely administered to patients with endotoxemia or sepsis.

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