Concentration-dependent roles for heparin in modifying liopolysaccharide-induced activation of mononuclear cells in whole blood

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Summary
In addition to their anticoagulant activity, unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH) have important immunomodulatory properties. However, different studies have reported conflicting pro- and anti-inflammatory effects in association with heparin. Moreover, the molecular basis for these heparin effects on inflammation remains unclear. It was the objective of this study to determine how UFH and LMWH regulate liopolysaccharide (LPS)-induced activation of human mononuclear cells in whole blood, and define the role of liopolysaccharide-binding protein (LBP) in mediating this effect. Whole blood was pre-treated with UFH or LMWH (0.1–200 IU/ml), prior to stimulation with LPS (10 ng/ml). After six hours, monocyte pro-inflammatory cytokine (interleukin (IL)-1β, IL-6, IL-8, and TNF-α) secretion was determined by plasma ELISA. Parallel experiments using THP-1 cell line and primary monocytes were performed under serum-free conditions, in the presence or absence of LBP (50–100 nM). Under serum-free conditions, heparin demonstrated dose-dependent anti-inflammatory effects, significantly reducing secretion of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) in response to LPS stimulation of THP-1 cells and primary monocytes. In contrast, in the presence of LBP, both UFH and LMWH demonstrated dose-dependent pro-inflammatory effects at all heparin concentrations. In ex-vivo whole blood experiments, pro-inflammatory effects (increased IL-1β and IL-8 following LPS-stimulation) of heparin were also observed, but only at supra-therapeutic doses (10–200 IU/ml). Our data demonstrate that in the absence of LBP, the direct effect of heparin on LPS-stimulated monocytes is anti-inflammatory. However, in whole blood, the immunomodulatory effects of heparin are significantly more complex, with either pro- or anti-inflammatory effects dependent upon heparin concentration.

Keywords
Heparins, inflammation, inflammatory mediators

Introduction
Heparin is a heterogeneous, highly sulphated glycosaminoglycan produced mainly by mast cells (1). The anticoagulant properties of heparin are well established (2). Heparin enhances anti-thrombin inhibition of critical serine proteases (notably factor Xa and thrombin respectively) required for coagulation (3). However, heparins can also bind to additional plasma proteins (including thrombin, platelet factor 4, heparin cofactor II, and fibronectin), and to the surfaces of both monocytes and endothelial cells (1, 4). In addition to their important anticoagulant properties, heparins possess other important biological functions (5).

In particular, heparins can modulate inflammatory responses. Previous studies have shown that heparin attenuates cytokine secretion (including interleukin (IL)-1β, IL-6, and TNF-α) from primary monocytes in serum-free medium following lipopolysaccharide (LPS) stimulation (6–8). These data are supported by a number of in-vivo animal studies that suggest the anti-inflammatory properties of heparins can be dissociated from their anticoagulant activity (9–11). In contrast, the anti-inflammatory effects of heparin on primary monocytes following LPS stimulation were not observed in presence of human serum (6). Furthermore, Heinzelmann and Bosshart recently reported that in whole blood experiments, heparin actually caused a significant
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Dose-dependent increase in monocyte secretion of the pro-inflammatory cytokine IL-8.

Lipopolysaccharide-binding protein (LBP) is a glycoprotein synthesised by hepatocytes and intestinal epithelial cells. It circulates in normal human plasma at a concentration of 5–10 μg/ml (100 nM), and rapidly binds free or membrane-associated LPS. LBP modulates physiological responses to LPS in a complex manner, dependent upon plasma LBP concentration. At normal plasma LBP concentrations, the capacity of LPS to activate monocytes through CD14 is enhanced following formation of LBP–LPS complexes. However, plasma LBP concentrations can increase significantly (60 μg/ml) in response to sepsis-related acute-phase responses. Interestingly, previous studies have demonstrated that at these higher LBP concentrations, the ability of LPS to induce mononuclear cell activation is significantly reduced. The critical role of LBP in mediating host inflammatory responses to LPS was observed in LBP null mice, which tolerated 1,000-fold higher LPS levels compared to wild-type controls. In the context of the divergent pro- and anti-inflammatory properties reported for heparin, it is noteworthy that both unfractionated (UFH) and low-molecular-weight heparin (LMWH) are able to bind to LBP with high affinities (K₄ ~ 0.55nM). Furthermore, binding of heparin to LBP in whole blood has been shown to facilitate the transfer of LPS to CD14, thereby enhancing LPS-induced activation of human monocytes.

In view of the wide and diverse clinical indications for heparin, it is clear that there are direct translational relevance to define how UFH and LMWH differentially regulate inflammatory responses to LPS. Furthermore, it is important to determine whether heparin at typical pharmacological concentrations exerts predominantly pro- or anti-inflammatory effects in vivo, and whether these immuno-modulatory effects are equivalent for both UFH and LMWH respectively. To address these questions, we have studied the ability of different heparin preparations over a range of different concentrations to modify LPS-induced activation of human mononuclear cells in either whole blood or under serum-free conditions. In addition, we have also investigated the hypothesis that LBP plays a critical role in mediating heparin effects on monocyte cytokine secretion, and that the concentration of LBP in human plasma may serve as an important determinant of the immuno-modulatory properties of heparin in the whole blood milieu.

Methods

Reagents

LPS (serotype 0127:B8) purified by phenol extraction from Escherichia coli was supplied by Sigma Chemicals (St. Louis, MO, USA). Recombinant LBP was obtained from Biometec (Greifswald, Germany). The THP-1 human monocyte suspension cell-line (American Type Culture Collection, Manassas, VA, USA) was initially cultured in medium consisting of RPMI 1640, supplemented with 10% foetal calf serum (FCS), L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml) (all Gibco/Invitrogen).

Human monocytes isolation and FACS staining

All blood donors were recruited in this study with informed consent in accordance with local ethical guidelines. Peripheral blood mononuclear cells (PBMCs) were obtained from citrate-anticoagulated venous blood by Lymphoprep density-gradient centrifugation (Nycomed, Oslo, Norway) as previously described. In brief, blood diluted in two parts in phosphate-buffered saline (PBS) was slowly layered over Lymphoprep; and platelets were removed after centrifugation at 400 g for 40 minutes (min). PBMC layers were collected and washed twice with PBS at 400 g for 10 min to eliminate further platelet contaminants and remaining Lymphoprep. Viable cells were counted using the trypan blue exclusion technique. The non-monocyte fraction of the PBMCs was then depleted using Monoclonal Antibody Cell Sorter (MACS) microbeads (Milteny Biotec, Bergisch Gladbach, Germany) conjugated to antibodies directed against lymphocytes, basophils, eosinophils and erythrocytes. Isolated monocytes were identified by flow cytometry (FACS Calibur, Becton Dickinson, Oxford, UK) following staining with phycoerythrin-conjugated mouse anti-human CD14 antibody (DakoCytomation Ireland Ltd, Galway, Ireland).

LPS-stimulation of THP-1 cells and primary monocytes

THP-1 cells or freshly prepared human monocytes were resuspended at a density of 1 x 10⁶ cells/ml in RPMI medium only, or supplemented with 10% heat inactivated foetal bovine serum and 10% autologous plasma, respectively. Cells were then treated with LPS (1–10 ng per 10⁶ cells) alone or in the presence of varying amount of heparin (0.1 – 200 IU/ml) and/or LBP (50 nM and 100 nM). UFH or LMWH was added to cells 15 min prior to stimulation with LPS. This incubation time was established using heparin time-course experiments using THP-1 cell line (data not shown), and LBP 5 min prior to heparin. Those samples not receiving LPS, UFH or LMWH received an equal volume of PBS. After 8 hours (h) incubation at 37°C and 5% CO₂, culture supernatants were aspirated and spun at 400 g for 5 min. Cell-free culture supernatants were then removed and stored at -80°C for subsequent cytokine measurements. All experiments were performed in triplicate, and all experimental data presented represent the mean and SEM of three independent experiments.

LPS-stimulation of mononuclear cells in whole blood

Human whole blood was obtained from healthy volunteers. Citrate anticoagulated venous blood was diluted 1:1 in RPMI and then treated with UFH or LMWH (0.1 – 200 IU/ml) 15 min prior to LPS (10 ng/ml). Again all experiments were performed in triplicate, and cell-free plasma supernatants stored at -80°C for subsequent analysis.

Enzyme-linked immunosorbent assay

Solid-based sandwich enzyme-linked immunosorbent assays (ELISAs) were carried out on cell culture supernatants using DuoSet ELISA Development Systems (R&D Systems, Minneapolis, MN, USA) for human TNF-α, IL-8, IL-6 and IL-1β according to the manufacturer’s instructions.
Constitutive secretion of IL-1β in whole blood was also profoundly increased following the addition of 10 ng/ml LPS (p<0.05). Moreover, we found that pre-treatment with either UFH or LMWH again caused a significant increase in LPS-induced secretion of IL-1β (Fig. 1). This novel effect of heparin on IL-1β secretion in whole blood was again dose-dependent and was observed at all concentrations greater than 10 IU/ml. Of note, LMWH (tinzaparin) was associated with significantly higher IL-1β responses than UFH at all heparin concentrations studied. Neither UFH nor LMWH had any effect on LPS-induced monocyte secretion of a number of other important pro-inflammatory cytokines including, TNF-α, IL-6, and IL-10 (data not shown).

**Effects of pharmacological heparin concentrations on LPS-induced monocyte activation in whole blood**

In contrast to the marked pro-inflammatory effects of high heparin concentrations, pre-treatment of whole blood with pharmacological heparin concentrations (0.1 to 1.0 IU/ml) (20) prior to LPS challenge was associated with a significant reduction in IL-8 secretion (p<0.05) (Fig. 2A). Moreover, both UFH and LMWH were associated with similar decreases in IL-8 response. Similarly, pre-incubation with heparin at either 0.1 or 1 IU/ml also resulted in significant reduction in IL-1β secretion (p<0.05) (Fig. 2B), but had no effect on monocyte secretion of other pro-inflammatory cytokines (TNF-α, IL-6, and IL-10) (data not shown).

**Effect of heparin on pro-inflammatory cytokine secretion from LPS-stimulated THP-1 cells and primary monocytes in vitro**

Under serum-free experimental conditions, constitutive IL-8 secretion from THP-1 cells was markedly increased following the addition of LPS (Fig. 3A). However pre-incubation with LMWH prior to LPS challenge resulted in a significant reduction in IL-8 response. Interestingly, this inhibitory effect of heparin was apparent at both low concentrations (1 IU/ml), and at high concentrations (200 IU/ml). Moreover, the anti-inflammatory effect was dose-dependent, and was also observed using UFH. Similarly, although LPS exposure resulted in a marked increase in IL-8 secretion by primary human monocytes cultured in vitro, this response was again significantly reduced by LMWH pre-incubation (Fig. 3B). Finally, in these serum-free experiments and again in contrast to our earlier experiments in whole blood, both UFH and LMWH caused a dose-dependent reduction in TNF-α secretion from THP-1 cells and primary monocytes following LPS stimulation (data not shown).

**Role of LBP in mediating the dose-dependent pro-inflammatory properties of heparin**

LBP is present in normal plasma and modulates host inflammatory responses to LPS. Under serum-free conditions, we observed that the elevation in IL-8 secretion by THP-1 cells following LPS challenge was significantly increased in the presence of 100 nm recombinant human LBP (from 2,200 to 3,850 pg/10^6 cells; p<0.05) (Fig. 4A). However, in addition, we also demonstrated that in the presence of LBP, the ability of LMWH to inhibit LPS-induced IL-8 secretion was no longer evident. Instead, LMWH in combination with LBP caused a dose-dependent increase in both IL-8 and IL-1β.
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secretion from LPS-activated THP-1. Similar results were obtained using UFH in combination with LBP (data not shown).

Although these results are consistent with previous findings in whole blood, it is interesting that in this in-vitro assay, even low physiological doses of heparin (0.1–1 IU/ml) were associated with significant pro-inflammatory effects. To further investigate this observation, we studied the effect of adding different doses of LBP to primary monocytes maintained in RPMI supplemented with 10% plasma (Fig. 4B). Under these conditions, the pro-inflammatory effects of LMWH were dependent upon the relative concentrations of both LBP and heparin respectively. Furthermore, the pro-inflammatory effects of low heparin concentration (0.1 IU/ml) were no longer observed at lower LBP concentration (50 nM).

**Discussion**

LPS is a large integral component of the outer membrane of Gram-negative bacteria, and plays a critical role in mediating the pathogenicity of these infections (21). Upon entry of LPS into the
human circulation, it is rapidly bound by LBP, and the resulting LPS-LBP complexes are recognised by the receptor CD14 on mononuclear cells (monocytes and macrophages) (13, 15). Binding of LPS to CD14 induces an intracellular signalling cascade involving NF-κB that in turn initiates secretion of inflammatory mediators (including TNF-α and a series of interleukins) (22–24). These cytokines up-regulate the host immune system, and thus eliminating Gram-negative infection. However excessive uncontrolled cytokine production can lead to dysregulation of the immune response, and to serious clinical consequences in the form of septic shock and multi-organ failure. Despite advances in antibiotic therapy and critical care therapy, severe Gram-negative sepsis continues to be associated with significant mortality rates in both children and adults (25). Consequently, the development of alternative therapeutic strategies that might be useful in interrupting the molecular and cellular pathophysiology underlying septic shock is of direct translational importance.

In keeping with previous reports, we have demonstrated that both UFH and LMWH can significantly down-regulate cytokine (TNF-α, IL-1β, IL-6 and IL-8) secretion in response to LPS-activation in vitro (6–8). This anti-inflammatory effect was evident using both the THP-1 cell line, and in purified primary human monocyte cultures. Furthermore, although the effect correlated with heparin concentration, a significant attenuation in secretion was also observed even at lower heparin concentrations consistent with those achieved during standard anticoagulation therapy. This reduction in pro-inflammatory cytokine secretion following LPS-activation may be explained at least in part by our recent observation that in a serum-free setting, heparin causes a dose-dependent reduction in nuclear translocation of the transcription factor NF-κB (19).

From the data presented in this study, we conclude that the effect of heparin on monocyte activation by LPS is significantly more complex in the setting of whole blood. Firstly, in contrast to the anti-inflammatory effects observed under serum-free conditions, we found that in whole blood, high concentrations of both UFH and LMWH exerted marked pro-inflammatory effects. However this effect of heparin was seen only with a subset of cytokines. Although secretion of IL-1β and IL-8 were both increased in response to LPS treatment, this effect did not extend to the other pro-inflammatory cytokines (TNF-α and IL-6). Secondly, we have also demonstrated that the effects of heparin in whole blood are entirely dependent upon heparin concentration. Although pro-inflammatory effects were observed with supraphysiological heparin concentrations (greater than 10 IU/ml), lower heparin concentrations had the opposite effect and significantly attenuated monocyte secretion of both IL-1β and IL-8. The mechanism underlying the anti-inflammatory effect of low heparin concentrations in whole blood remains unclear. However, interestingly, these divergent and paradoxical effects of heparin mirror those previously reported for LBP, which exhibits a concentration-dependent ability to either neutralise or enhance LPS-induced activation of mononuclear cells (16).

A recent study by Heinzelmann and Bozart demonstrated that heparin binds to LBP, and thereby increases LBP complex formation with LPS (12). In order to elucidate the mechanism(s) in whole blood responsible for the paradoxical effects of different heparin concentrations, we therefore investigated the potential role of LBP. We found that in the presence of recombinant human LBP, the ability of heparin to attenuate THP-1 cytokine secretion in response to LPS was entirely reversed. Instead, both UFH and LMWH caused dose-dependent increased secretion of both IL-1β and IL-8. These data illustrate the critical importance of LBP in regulating the immuno-modulatory properties of he-
parin. However, it is interesting to note that there remain important differences between the effects of heparin in whole blood compared to their effects in serum-free conditions even with added LBP. In whole blood, standard therapeutic heparin concentrations of UFH and LMWH both significantly attenuated IL-1β and IL-8 responses. In contrast, in serum-free medium with LBP, therapeutic heparin concentrations were associated with increased cytokine secretion. These findings demonstrate that the immuno-modulatory effects of heparin in vivo are complex and not entirely mediated through LBP. Previous studies have shown that heparin can directly influence activation of the complement pathway, and also that antithrombin can suppress inflammatory responses to LPS (6, 26). Furthermore, LBP is only one of a family of LPS-binding proteins that circulate in normal plasma (27).

These data clearly demonstrate that, aside from their anticoagulant properties, therapeutic concentrations of heparin may also have clinically important effects on inflammation. Indeed, several clinical trials have suggested that heparin may be a useful additional immunosuppressive therapy in patients with inflammatory bowel disease (28–30), rheumatoid arthritis (31), or asthma (32). However, from our findings it is clear that the immuno-modulatory effects of heparin are paradoxical in nature dependent upon plasma concentration. In addition, we have demonstrated that plasma LBP concentration is a critical determinant in regulating the differential pro- and anti-inflammatory effects of heparin in the context of Gram-negative infection. This observation is of physiological significance in view of the marked increase in plasma LBP levels typically associated with severe sepsis syndrome. However, further studies will be important to determine whether these complex effects of heparin are observed only in the setting of LPS, or are more widely applicable.

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Abbreviations

UFH, unfractionated heparin; LMWH, low-molecular-weight heparin; IL, interleukin; LPS, lipopolysaccharide; LBP, lipopolysaccharide-binding protein; TNF, tissue necrosis factor.

References