Heparin Blunts Endotoxin-Induced Coagulation Activation

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Background—Lipopolysaccharide (LPS) is a major trigger of sepsis-induced disseminated intravascular coagulation (DIC) via the tissue factor (TF)/factor VIIa-dependent pathway of coagulation. Experimental endotoxemia has been used repeatedly to explore this complex pathophysiology, but little is known about the effects of clinically used anticoagulants in this setting. Therefore, we compared with placebo the effects of unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH) on LPS-induced coagulation.

Methods and Results—In a randomized, double-blind, placebo-controlled trial, 30 healthy male volunteers received LPS 2 ng/kg IV followed by a bolus-primed continuous infusion of UFH, LMWH, or placebo. In the placebo group, activation of coagulation caused marked increases in plasma levels of prothrombin fragment F1+2 (P<0.01) and polymerized soluble fibrin, termed thrombus precursor protein (TpP; P<0.01); TF-positive monocytes doubled in response to LPS, whereas levels of activated factor VII slightly decreased and levels of TF pathway inhibitor remained unchanged. UFH and LMWH markedly decreased activation of coagulation caused by LPS, as F1+2 and TpP levels only slightly increased; TF expression on monocytes was also markedly reduced by UFH. TF pathway inhibitor values increased after either heparin infusion (P<0.01). Concomitantly, factor VIIa levels dropped by >50% at 50 minutes after initiation of either heparin infusion (P<0.01).

Conclusions—This experimental model proved the anticoagulatory potency of UFH and LMWH in the initial phase of experimental LPS-induced coagulation. Successful inhibition of thrombin generation also translates into blunted activation of coagulation factors upstream and downstream of thrombin. (Circulation. 1999;100:2485-2490.)

Key Words: heparin ■ endotoxin ■ coagulation ■ anticoagulants ■ fibrin

Even with appropriate antimicrobial and supportive care, many patients die of sepsis, which makes strategies for prevention and more effective treatment of critical importance. During sepsis, bacterial mediators such as lipopolysaccharide (LPS) trigger the generation of microthrombi and the consumption of coagulation factors and their endogenous inhibitors, thereby leading to disseminated intravascular coagulation (DIC). LPS stimulates endothelial cells and blood monocytes to express tissue factor (TF); TF then forms a highly procoagulant complex with activated factor VII (FVIIa), which initiates the coagulation cascade during endotoxemia. Ongoing DIC causes changes in plasma levels of all coagulation factors: clinical studies reported decreased levels of FVIIa and increased levels of soluble TF, prothrombin fragment (F1+2), and soluble fibrin, resulting in increases of fibrin split products such as d-dimer. In the clinical setting, inhibition of coagulation with low doses of unfractionated heparin (UFH) or low-molecular-weight heparin (LMWH) has been recommended by some authors, although no consensus exists on the clinical efficacy of either drug.

Heparin enhances inactivation of thrombin and factor Xa via antithrombin III but also increases plasma levels of endogenous TF pathway inhibitor (TFPI). Animal data suggest that enhancement in TFPI activity represents an upstream and even more specific anticoagulatory action in LPS-induced coagulation. Injection of LPS into human volunteers provides a standardized model to study the pathogenesis of the initial phase of systemic coagulation activation. Therefore we used this human model to elucidate whether clinically applied doses of UFH or LMWH impede thrombin generation during endotoxemia compared with placebo. We also aimed to delineate how blunted thrombin generation affects coagulation factors upstream and downstream of thrombin.

Methods
Study Design and Study Subjects
The study was approved by the Institutional Ethics Committee. Written informed consent was obtained from all participants. The
study was a randomized, double-blind, placebo-controlled study in 3 parallel groups of 30 healthy male subjects (n=10 per group).

Mean age of the volunteers was 28±6 years, and body mass index averaged 23.5±2.1 kg/m². Determination of health status included medical history, physical examination, laboratory parameters, and virological and drug screening. In addition, study subjects were tested for hereditary thrombophilia. Exclusion criteria were previous exposure to heparin and recent intake of medication, including nonprescription medication.

Study Protocol

Volunteers were admitted to the study ward at 8 AM after an overnight fast. Throughout the entire study period, subjects were confined to bedrest. A 5% glucose infusion (Leopold Pharma) was started at 8:15 AM and continued over 8.5 hours at 3 mL·kg⁻¹·h⁻¹ to maintain adequate blood glucose levels and urinary output. Concomitant with the onset of infusion, participants of the trial received 500 mg of paracetamol (Paracetamol Genericon Pharma), which alleviates subjective symptoms without compromising the host response.³¹ Thirty minutes thereafter, all subjects received a bolus of LPS 2 ng/kg IV (national reference endotoxin, Escherichia coli; USP Convention Inc, Rockville, Md). Ten minutes after LPS infusion, study subjects in the UFH group received 80 IU/kg (Heparin Immuno, Immuno Ag) followed by a continuous UFH infusion, study subjects in the UFH group received 80 IU/kg (Heparin Immuno, Immuno Ag), and study subjects in the LMWH group received Fragmin (Pharmacia & Upjohn) 40 IU/kg followed by continuous infusion of 15 IU·kg⁻¹·h⁻¹ for 6 hours. In the placebo group, identical volumes of saline were infused.

The doses of UFH and LMWH used in the present trial have previously been reported to be equipotent in preventing fibrin deposition during hemodialysis.¹⁶ Furthermore, the duration of hemodialysis, ie, 4 hours, is comparable to the time span of coagulation activation in our endotoxin model. Finally, the dose of LMWH is the highest currently licensed intravenous dose.

Sampling

Blood samples were collected into citrated evacuated-container tubes by venipuncture at 30 minutes before infusions and 1, 2, 3, 4, 6, and 24 hours after intravenous LPS (Figure 1) (final concentration 0.13 mmol/L sodium citrate, Vacutainer, Becton Dickinson). Citrated plasma samples were processed immediately by centrifugation at 2000g at 4°C for 15 minutes and stored at −80°C before analysis.

Blood Cell Counts

Monocyte counts were calculated from scatter histograms obtained with a flow cytometer (Becton Dickinson), because morphological analysis revealed that monocyte levels measured with the cell counter were spuriously high.¹⁵ Flow cytometry was performed by analysis of 20,000 gated events, as previously described.¹⁷ Because all samples required immediate processing to avoid artificial activation of leukocytes, cells were stained before and 2, 6, and 24 hours after LPS infusion. The fluorescein-isothiocyanate–coupled anti-TF monoclonal antibody was purchased from American Diagnostics Inc.¹⁸

Analyses

Plasma levels of TFPI were determined with a 2-stage chromogenic substrate assay.¹⁹ Values were compared against pooled plasma from 48 normal individuals, and TFPI activity was calculated as a percentage of this reference value.

The following commercially available assays were used: FVIIa (Staclot VII-rTF assay, Diagnostica Stago; normal range 25 to 113 mU/mL); factor VIIc (FVIIc; Diagnostica Stago; normal range 60% to 180%), factor VII antigen (FVII:Ag; Asserachrom VII:Ag, Diagnostica Stago; normal range 76% to 123%); and prothrombin fragment F₁₋₂ (Behring; normal value <1.9 nmol/L).²¹ To quantify soluble fibrin, 2 tests with different principles were used. First, we used a chromogenic assay that used the potential of fibrin to convert plasminogen to plasmin (Coaest, Chromogenix; normal range 25 to 75 arbitrary units).¹⁰,²² Second, we used an enzyme immunoassay (EIA) for polymers of soluble fibrin, termed thrombus precursor protein (TTP, American Biogenetic Sciences; normal values <6 µg/mL). The antibody of this assay does not cross-react with fibrinogen, desAAFibrin, or d-dimer.²³ The lack of cross-reactivity between TTP and d-dimer was confirmed by our own experiments using d-dimer standards at concentrations up to 1000 ng/mL in vitro (data not shown).

Fibrinolysis was assessed with the following assays: EIA tissue plasminogen activator (tPA), which measures total tPA antigen, ie, free molecules and molecules complexed to plasminogen activator inhibitor (PAI) (t-PA, Chromogenix; normal range 1 to 12 ng/mL), and EIA PAI, which measures free active molecules not complexed with tPA (Technoclone; normal range 10 to 30 ng/mL); the fibrin split product d-dimer (Boehringer Mannheim; normal values <400 ng/mL) results from fibrinolytic digestion of fibrin.

Antithrombin levels (STA antithrombin, Diagnostica Stago; normal range 75% to 125%) were determined on the STA analyzer (Stago). With the same analyzer, anti-Xa activity was assessed (Rotachrom heparin and Rotachrom heparinol based reagents/ LMWH, Stago; detection threshold of both assays: anti-Xa 0.1 IU/mL; calibration with specific reagents).

Data Analysis

Data are expressed as mean and 95% CI or the range. Owing to nonnormal distribution, nonparametric tests were applied. Comparisons within groups were done by Friedman ANOVA and Wilcoxon signed rank test for post hoc comparisons. For comparisons between groups, the Kruskal-Wallis ANOVA was applied, followed by Mann-Whitney U test. Because most measured parameters are interdependent and to limit statistical comparisons to a reasonable amount, F₁₋₂ generation was determined a priori as the main outcome variable. Post hoc comparisons were restricted to times of peak values, whereas all other data are presented in a descriptive manner (95% CI).

Results

Baseline data are presented in the Table. All parameters were significantly different between groups with the exception of 25% lower levels of FVII:Ag in the heparin group (P = 0.003 versus placebo) and 35% lower levels of soluble fibrin in the UFH group than in the LMWH group (P = 0.007 versus LMWH).
TF Expression on Monocytes

After LPS infusion, monocyte counts fell to undetectable values after 2 hours. At 6 hours, monocyte counts averaged $0.30 \times 10^9/L$ (range 0.08 to 0.92) in the placebo group, $0.15 \times 10^9/L$ (range 0.02 to 0.35) for UFH, and $0.13 \times 10^9/L$ (range 0.08 to 0.30) for LMWH. Neither the frequency nor the degree of monocytopenia at 6 hours was different between groups ($P > 0.05$). At baseline, 9% (95% CI 7.5% to 11.1%) of circulating monocytes were positive for TF. Owing to the monocytopenia, this parameter could not be evaluated at 2 hours. Furthermore, monocytopenia was still present in 50% of the subjects in each of the 3 groups at 6 hours, which excluded these subjects from evaluation of TF expression by flow cytometry. In the placebo group, TF-positive monocytes doubled at 6 hours. In contrast, no increase of TF-positive monocytes occurred in the UFH group at 6 hours ($P = 0.028$ versus placebo), and the increase in TF positivity was blunted in the LMWH group (data not shown).

TFPI, Anti-Xa, and Antithrombin III

LPS infusion did not change TFPI plasma levels in the placebo group. As expected,12 TFPI values increased almost 3-fold after administration of UFH or LMWH ($P < 0.01$ versus placebo; Figure 1). Anti-Xa values rose sharply in the UFH group to peak values of 1.6 U/mL (95% CI 1.4 to 1.9 U/mL) at 60 minutes after LPS infusion. In the LMWH group, anti-Xa levels were only 50% of values obtained in the UFH group during the first 4 hours of infusion ($P < 0.05$ versus UFH; Figure 1). At 6 hours, however, anti-Xa activity was equal in the UFH and LMWH groups. Antithrombin III values declined by 3.6% (95% CI 0.5% to 7.7%) in the placebo group, by 8.5% (95% CI 5.2% to 11.8%) in the UFH group, and by 1.3% (95% CI 1.8% to 4.5%) in the LMWH group 3 hours after LPS infusion (data not shown).

FVIIa, FVIIc, and Factor VII Antigen

FVIIa levels decreased steadily after LPS infusion in the placebo group and were $\approx 25\%$ lower at 24 hours ($P < 0.01$ versus baseline; Figure 1). In contrast, FVIIa levels dropped by $> 50\%$ at 50 minutes after start of either heparin infusion. Levels of FVIIc exhibited a similar pattern (data not shown). Plasma levels of FVII:Ag decreased by $\approx 20\%$ in all groups at 24 hours, although baseline values of FVII:Ag were different in the 3 groups (Table).

F$_{1+2}$, TpP, and Soluble Fibrin

Plasma levels of F$_{1+2}$ increased 10-fold in the placebo group ($P < 0.005$ versus baseline; Figure 2). In contrast, UFH infusion completely abolished F$_{1+2}$ generation, whereas F$_{1+2}$

Figure 2. Plasma levels (mean ± SEM) of F$_{1+2}$ (top) before and after LPS infusion (2 ng/kg) in human volunteers receiving either placebo (○), UFH (●), or LMWH (■) (n = 10 per group). Plasma levels of soluble fibrin (SF; middle) and TpP (bottom) are also shown. *$P < 0.01$ vs baseline; #P < 0.01 between groups.
predicted adverse outcome in patients with unstable angina.27,28

Figure 2). In clinical routine, this represents the earliest time to coagulation induction during renal replacement therapy.

To the best of our knowledge, this is the first trial to compare the effects of UFH and LMWH versus placebo in LPS-induced coagulation. Whereas UFH entirely blocked F$_{1+2}$ generation, LMWH only partially inhibited the increase in F$_{1+2}$ generation (Figure 3). However, we used the highest currently licensed dose of LMWH, which amounted to a total of $\approx$ 10 000 U of LMWH over 6 hours (Figure 1). This dose prevented coagulation induction during hemodialysis.16,32 Although anti-Xa activity was lower in the LMWH group than in the UFH group during the initial 4 hours of infusion, no difference between the groups was observed at 6 hours (Figure 1). In clinical routine, this represents the earliest time when inhibition of factor Xa activity is measured after initiation of therapy. This difference of anti-Xa activity is clinically relevant, because it suggests that higher doses of LMWH may be needed to completely blunt LPS-induced coagulation.

Discussion
In clinical practice, UFH and LMWH are used for the treatment of DIC, although no consensus exists regarding choice or dose of the drug.1,2 Furthermore, the heterogeneity of DIC and the severity of concomitant disease may have precluded the development of successful therapeutic approaches thus far. Infusion of small doses of LPS in human volunteers has emerged as a valuable model to safely study endotoxin-induced coagulopathy.24,25 Therefore, we set out to investigate the potency of UFH and LMWH in LPS-induced coagulation.

In accordance with our own previous findings,18 TF-positive monocytes doubled after LPS infusion in the placebo group. The clinical relevance of our finding is supported by a report that high tissue thromboplastin activity on monocytes predicted adverse outcome in patients with Neisseria meningitidis infection.26 Now, we report for the first time that UFH abrogates the LPS-induced increase in TF-positive monocytes in vivo. These data agree with the inhibiting effects heparin exerts on LPS-induced TF mRNA production in vitro and with a recent clinical trial, which showed that heparin reduces TF plasma levels and monocyte procoagulant activity in patients with unstable angina.27,28

TF forms a highly procoagulant complex together with FVIIa, which prompted us to study the regulation of FVIIa levels. FVIIa levels declined steadily in the placebo group and were minimal 24 hours after LPS infusion. In sharp contrast, FVIIa fell sharply within 1 hour after initiation of heparin infusion and returned to baseline levels 24 hours later (Figure 2). Interestingly, Mesters et al.8 recently reported a correlation between low FVIIa levels and poor outcome in septic patients with DIC. Unfortunately, no information was provided on concomitant treatment with either heparin, with or without hemofiltration. On the basis of our findings, it appears that FVIIa may only indicate unfavorable prognosis if FVIIa levels are determined before heparin infusion. Along these lines, we recently reported a similar decrease of FVIIa levels in heparin-infused volunteers who were not subjected to coagulation activation.29 Our findings therefore challenge the concept of the predictive value of FVIIa levels in critically ill patients, because these patients are very likely to receive heparins during renal replacement therapy.
thrombin generation. In addition, it underlines that factor Xa serves as a major trigger of thrombin formation in endotoxemia. Taken together, our data show that low-dose LMWH administration cannot fully prevent LPS-induced thrombin formation.

As a consequence of thrombin generation, one may expect a marked increase in soluble fibrin in the placebo group. Accordingly, we found a 6-fold increase in the TpP EIA that used an antibody against polymerized soluble fibrin (Figure 2). This antibody against soluble fibrin does not cross-react with fibrinogen, batroxobin-digested fibrinogen (ie, desAAfibrin), or d-dimer.23 Thus, we confirm our previous finding that TpP levels increase during endotoxemia.18 We also showed that UFH and LMWH equally blunted the TpP increase after LPS (Figure 2). In contrast, using a chromogenic assay based on fibrin-mediated conversion of plasminogen to plasmin,22 we found no increase in any study subject. Although this confirms our previous results with twice the dose of LPS, our findings are at variance with clinical trials10,22 that reported increases in soluble fibrin with similar assays. Differences in sensitivity between chromogenic assays and EIA for soluble fibrin have been described previously, particularly at soluble fibrin levels <10 μg/mL,33,34 and are a likely explanation for the discrepancies between the functional assay and the TpP assay found in our trial (Figure 2).

In good agreement with previous reports,35 we found a parallel release of tPA and PAI-1 after 2 hours, which resulted in increased total tPA antigen levels and almost unchanged free active PAI-1. At 3 hours after infusion and during the subsequent 2 hours, obviously higher amounts of PAI-1 were released into the circulation, because total tPA antigen levels did not rise further, whereas the amount of free active PAI-1 markedly increased. These phenomena were similar in the 3 groups (Figure 3). Increased d-dimer levels, representing actual fibrinolytic activity, were only seen in the placebo group when fibrin was formed (Figure 2). Plasma levels of TpP and d-dimer changed in parallel, which suggests that fibrin formation is the most relevant fibrinolytic stimulus. Differences in plasma levels of TpP (ie, cross-linked soluble fibrin) between treatment groups affected neither plasminogen conversion in the functional soluble fibrin assay nor plasma levels of tPA and PAI-1 (Figures 2 and 3). This indicates that during experimental endotoxemia, TpP is not a major mediator of tPA plasma levels in this model, possibly because tumor necrosis factor-α has maximally enhanced tPA and PAI-1 release.7

We conclude that UFH blocks the upregulation of TF expression on circulating monocytes, increases TFPI release, decreases FVIIa levels, and blunts generation of F1 + 2. TpP, and d-dimer, whereas it has no effect on TPA and PAI-1 release. LMWH at the currently used doses was a less effective inhibitor of thrombin generation in experimental endotoxemia.

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Heparin Blunts Endotoxin-Induced Coagulation Activation

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