Thrombelastography for the monitoring of lipopolysaccharide induced activation of coagulation

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Summary

During Gram-negative sepsis, lipopolysaccharide (LPS) activates toll-like receptor (TLR) 4 and induces complex responses of immune system and haemostasis. In the present study we investigated whether thrombelastography is suitable to monitor the LPS-induced activation of coagulation. Whole blood samples from healthy volunteers were incubated with LPS for various incubation periods (0–5 hrs), thereafter rotation thrombelastography was performed. Incubation of whole blood (> 3 h) with LPS markedly reduced clotting time; after 5 hrs the variable was reduced from 459 ± 39 sec to 80 ± 20 sec while the other thrombelastography variables (angle α, clot formation time, maximal clot formation) remained unaltered. EC50 of the LPS-effect on whole blood clotting time was 18 µg/ml. In isolated leukocytes, diluted in platelet poor plasma, far lower LPS-concentrations were effective: 10 ng/ml LPS reduced clotting time from 439 ± 68 sec to 200 ± 56 sec. Experiments with the protein synthesis inhibitor cycloheximide and active site-inhibited factor VIIa revealed that LPS exerts its effects via the synthesis of tissue factor. Addition of tissue factor to whole blood samples revealed that a concentration of 100 fmol/l can be detected using thrombelastography. In whole blood samples the tissue factor concentration induced by LPS amounted up to 12 pmol/l. In summary, thrombelastography proved to be a sensitive and reliable tool for the determination of LPS-induced tissue factor mediated activation of haemostasis in whole blood samples.

Keywords

Human whole blood, lipopolysaccharide, thrombelastography

Thromb Haemost 2006; 95: 557–61

Introduction

During Gram-negative sepsis, activation of the coagulation system is critically involved in the pathophysiology and clinical course of the disease (1, 2). Lipopolysaccharide (LPS) from invading bacteria stimulates toll-like receptor 4 (TLR-4) as the principle LPS-receptor and thus induces complex responses of both immune system and haemostasis (3, 4).

Although the clinical importance of the coagulation system has been recognised from the results of the PROWESS study (5), there is no bedside method to monitor the hypercoagulable state during sepsis. Since coagulopathy in sepsis is, at least in part, due to expression of tissue factor on monocytes (6–11), a cell based coagulation monitoring system might be valuable.

In the present study we investigated whether LPS-induced activation of coagulation in whole blood samples can be monitored by use of thrombelastography and examined the mechanism of the endotoxin’s action.

Material and methods

Blood sampling

Venous blood was drawn from the antecubital vein of healthy volunteers. After discarding the first 2 ml, blood was collected in one tenth volume of citrate (3.8%, Becton Dickinson Vacutainer®) and samples were immediately used for the experiments. The ethical principles as set out in the Declaration of Helsinki were honoured in the present study.

Fractionation of whole blood samples

To obtain platelet poor plasma, whole blood aliquots were centrifuged at 2000 x g for 20 minutes. Absence of both leukocytes and platelets in platelet poor plasma was verified by transmission microscopy. Preparation of leukocytes was performed as recently described (12). In short, 30 ml blood was drawn in a syringe containing 5 ml of ACD-A. Thereafter, 6 ml hydroxy-ethyl starch (6%) was added and red blood cells were allowed to sedi-
ment for 60 minutes. The cell rich supernatant was then centrifuged at 150 x g for 5 minutes. Thereafter, leukocyte pellet was reconstituted in phosphate buffered saline to 30,000 cells/µl and incubated with LPS. Before thrombelastography, platelet poor plasma was added to the leukocyte suspensions to obtain a leukocyte count of 7,500 cells/µl.

Incubation of blood components with LPS
In all experimental series, incubation of blood or blood fractions was performed at 37°C. i) Whole blood was incubated with LPS (1 mg/ml) or vehicle (NaCl 0.9%) for different time intervals (0–5 hrs). ii) Whole blood was incubated with different concentrations of LPS (0.005 – 1 mg/ml) or vehicle for 4 hrs. iii) A standard curve of clotting time for exogeneously administered tissue factor (Thromborel-S) was generated (13). iv) LPS or vehicle was added to whole blood, leukocyte suspensions and platelet poor plasma, respectively, incubation time was 4 hrs in each group. Furthermore, clotting time of platelet poor plasma, obtained from whole blood previously incubated with LPS (1 mg/ml) for 4 hrs, was determined. v) Whole blood samples were incubated with cycloheximide, dexamethasone, or vehicle for 30 minutes. Thereafter, LPS (1 mg/ml) or vehicle was added to the pretreated samples. In a further series, active site-inhibited factor VIIa (50 µg/ml) was added to whole blood samples which had been incubated with LPS (1mg/ml) or vehicle for 4 hrs (14). Subsequent to the above specified incubation steps, all samples were immediately subjected to thrombelastography.

Thrombelastography
Citrated samples were recalcified with calcium chloride and subjected to rotational thrombelastography (Roteg 5, Pentapharm, Munich, Germany), a modification of the original thrombelastography method. In the Roteg system a pin is fixed on the tip of a rotating shaft. The shaft rotates back and forth (+/- 4.75 °) and is connected with a spring for the optoelectric measurement of elasticity. The four standard variables obtained from a Roteg-thrombelastogram were analysed. The clotting time (CT) is defined as the period of time from initiation of test to initial fibrin formation and is inhibited by direct and indirect thrombin inhibitors. The clot formation time (CFT) measures the time from beginning of clot formation until the amplitude of the thrombelastogram reaches a width of 20 mm. The angle α represents the kinetics of fibrin build-up and cross-linking. The maximal amplitude (MCF) reflects the strength of the clot which is dependent on number and function of platelets and its interaction with fibrin.

Materials
LPS (Escherichia coli; serotype 0.111:B4) was obtained from Sigma-Aldrich, Germany. Tissue factor (Thromborel-S) was obtained from Dade-Behring, Germany. Hydroxy-ethylstarch (Voluven) was purchased from Fresenius Kabi, Germany. Active site-inhibited factor VIIa (ASIS) was a generous gift from Novo Nordisk, Denmark. All other reagents were of analytical grade.

Statistics
All data are given as the mean and standard deviation. For statistical evaluation the Mann-Whitney-Test was used and statistical significance was assumed with p-values below 0.05 (Openstat). For the curve fits (Figs. 4, 5) a four parameter Hill plot was used (Sigma Plot, SPSS Inc, USA).

Results
Thrombelastography detects LPS induced activation of coagulation in whole blood
As shown by the representative thrombelastograms in Figure 1, incubation of whole blood with LPS (1 mg/ml, 4 hrs) led to a marked shortening of clotting time (CT) by 70%, while CFT, MCF and angle α were not affected. The time course of the LPS-induced reduction in CT, shown in Figure 2, demonstrates that CT decreased as the incubation period with LPS increased. A maximal reduction of the variable from 578 +/- 94 sec (n=5) to 80 +/- 20 sec (n=5) was observed after 5 hrs of incubation with LPS. Incubation of whole blood (0 – 5 hrs) in absence of LPS did not affect the CT. In contrast to its marked effects on CT, LPS had little or no effect on MCF, CFT and angle α (Fig. 3). Therefore, these three variables are not shown in the remaining experimental series. In a further series, whole blood samples were incubated with varying concentrations of LPS (0.005 – 1 mg/ml) for 4 hrs (Fig. 4). The EC50 of the LPS induced shortening of clotting time was 18 µg/ml.

Figure 1: Representative thrombelastograms demonstrating the effect of LPS (1 mg/ml; 4 hrs incubation) or vehicle on thrombelastography. The clotting time (CT) represents the time from recalcification of whole blood sample to onset of coagulation. The maximal clot firmness (MCF) characterizes the physical strength of the clot. Angle α and clot formation time (CFT) define the kinetics of clot formation.

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Comparison of LPS and tissue factor mediated effects on coagulation

To evaluate the pathophysiological importance of LPS on coagulation, we generated a standard curve using tissue factor standards. As shown in Figure 5, tissue factor (100 fmol/l – 1 nM) markedly shortened CT. The maximal shortening of clotting time induced by LPS, which was 80 ± 20 secs (see Fig. 4), is comparable to 12 pmol/l tissue factor.

Cells are involved in the LPS-induced activation of coagulation

To investigate the role of cellular blood components for the LPS-induced activation of coagulation different blood fractions were incubated with the endotoxin for 4 hours. LPS significantly reduced CT in whole blood and leukocyte suspensions but did not affect CT in platelet poor plasma (Fig. 6A-C). Notably, in leukocyte suspensions a final LPS-concentration of 10 ng/ml reduced clotting time by 54%. To find out whether the procoagulant factor induced by LPS is soluble or cell-bound, CT was determined in platelet poor plasma obtained from whole blood incubated with LPS first (1 mg/ml, 4 hrs). The reduction of CT in this series demonstrates that a soluble factor is released (Fig. 6D).

Effects of cycloheximide, active site-inhibited factor VIIa (ASIS) and dexamethasone

In a further experimental series, we determined whether gene induction and protein synthesis are necessary for the LPS induced activation of coagulation. Whole blood was incubated with cycloheximide (35 µM) followed by 4 hrs of incubation with LPS (1 mg/ml). The protein synthesis inhibitor completely inhibited the effects of LPS on coagulation (Fig. 7A). To identify the LPS induced activator of coagulation, experiments with the tissue factor blocker ASIS were performed. ASIS, which was added to whole blood samples 5 minutes before thrombelastography, completely inhibited the LPS induced shortening of CT (Fig. 7B). In contrast to the latter agents, dexamethasone (100 µM) ap-
plied under otherwise identical experimental conditions, did not affect the LPS-induced shortening of CT (data not shown).

Discussion

It is well recognised that immune system and coagulation cascade are both activated in sepsis. The coagulopathy in sepsis is crucial as it is thought to lead to tissue ischaemia, hibernation, stunning and, thus, to organ dysfunction (15, 16). According to this notion the natural inhibitors antithrombin, tissue factor pathway inhibitor (TFPI) and activated protein C demonstrated favorable effects in experimental sepsis and septic patients, respectively (1, 5). As the underlying pathomechanisms for the coagulopathy in sepsis an activation of the extrinsic as well as the intrinsic coagulation system has been suggested (17–20). During sepsis the expression of tissue factor on the surface of monocytes and its release into the blood stream is thought to be of special importance since tissue factor is normally restricted to the extravascular space (6–11).

Thrombelastography was used in the present study since global coagulation characteristics of whole blood samples (including all cellular components) can be determined with this method. Thrombelastography, introduced by Hartert in 1949 (21), is established in the setting of cardiopulmonary bypass surgery and orthotopic liver transplantation (22–25). Similar to coagulometers commonly used in laboratory coagulation assays, thrombelastography measures the time until a clot is formed (clotting time, CT). In addition, however, the physical strength of a clot as well as the kinetics of clot formation are determined. The respective variables are clot formation time (CFT, period from onset of coagulation to a certain clot strength), maximal clot firmness (MCF) as well as the angle $\alpha$.

In the present study, LPS markedly shortened CT (Figs. 1, 2) from 459 ± 39 sec to 80 ± 20 sec but did not change CFT, MCF or angle $\alpha$. The onset of the LPS induced activation of coagulation in our study was delayed which argues for a complex mechanism of action. LPS did not alter the clotting time of platelet poor plasma suggesting a cell mediated effect. When, however, LPS-incubated leukocytes were suspended in platelet poor plasma, a marked shortening of clotting time was determined (Fig. 6). Thus, a critical involvement of leukocytes is obvious. The procoagulant effects of LPS was demonstrated to be mediated by de novo synthesis of tissue factor, since cycloheximide and active site-inhibited factor VIIa, respectively, completely inhibited the LPS induced shortening of clotting time. The finding, shown in Figure 6D, that the LPS induced procoagulant activity is, at least in part, soluble can most likely be explained by tissue factor bearing microparticles (11).

The EC$_{50}$ of the LPS-effect in the present whole blood experiments was 18 µg/ml and this values corresponds well to the concentration range of other whole blood studies (26–31). In contrast, in cell culture experiments LPS is often administered in far lower concentrations (32, 33). In accordance to these citations, our experiments with leukocyte suspensions demonstrate that 10 ng/ml LPS reduced CT from 439 ± 68 sec to 200 ± 56 sec (Fig. 6B). The differences in LPS sensitivity between whole blood experiments and leukocyte suspensions can be explained by the physicochemical properties of LPS: The endotox-

Figure 6: Effects of LPS on clotting time in whole blood (LPS 1 mg/ml), leukocyte suspensions (10 ng/ml), platelet poor plasma (PPP; LPS 1mg/ml) and platelet poor plasma derived from whole blood samples pre-incubated with LPS (1 mg/ml). Incubation time with LPS was 4 hrs in all experiments. Mean ± SD; n=5. $^p<.05.$

Figure 7: Effects of cycloheximide and active site-inhibited factor seven on on clotting time of whole blood. A) Effects of cycloheximide (35 µM) pretreatment on clotting time of whole blood incubated under control conditions (CON) or in the presence of LPS (1 mg/ml). Mean ± SD; n=5. $^p<.05.$ B) Effects of active site-inhibited factor seven (50 µg/ml) on the clotting time of whole blood samples previously incubated with LPS (1mg/ml, 4 hrs) and vehicle, respectively. Mean ± SD; n=6. $^p<.05.$
in is never free in biological fluids but constantly attached to a large panel of peptides (34, 35). Accordingly, low plasma LPS levels of 300 pg/ml are measured in septic patients, while in a recent study LPS-concentration measured in erythrocytes was 77 ± 26 µg/ml (28, 36).

The tissue factor standard curve (Fig. 5) demonstrates that as low as 100 fmol/l tissue factor can be measured using thrombelastography. Since maximal LPS-induced tissue factor was 100-fold higher in the present study, an adequate sensitivity of thrombelastography can be assumed.

In conclusion, the results of the present study demonstrate that thrombelastography is a sensitive and reliable tool for the assessment of the LPS-induced, tissue factor mediated activation of coagulation. In further studies the value of thrombelastography in patients with Gram-negative sepsis has to be investigated.

References