Interaction between inflammation, coagulation and fibrinolysis during infection

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Chapter 12

Inhibition of plasmin activity by tranexamic acid does not influence inflammatory pathways during human endotoxemia

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Abstract

Plasmin activates several pro-inflammatory pathways at the cellular level in vitro. LPS administration to healthy humans results in a rapid generation of plasmin activity, accompanied by activation of a number of inflammatory systems. To determine the role of early plasmin activity in LPS-induced inflammation in vivo, 16 healthy males received an intravenous bolus injection with LPS (from *Escherichia coli*, 4 ng/kg) directly preceded by a 30-min intravenous infusion of tranexamic acid (2 g, n = 8), a plasmin activation inhibitor, or placebo (n = 8). LPS injection induced marked increases in the plasma levels of D-dimer and plasmin-α2-antiplasmin complexes, indicative for plasmin activation and generation respectively, which were strongly attenuated by tranexamic acid (both P < 0.01 vs placebo). However, tranexamic acid did not influence LPS-induced coagulation activation, granulocytosis, neutrophil activation (expression of CD11b, CD66b and L-selectin) or degranulation (plasma concentrations of elastase-α1-antitrypsin and bactericidal permeability-increasing protein), endothelial cell activation (plasma levels of von Willebrand factor and soluble E-selectin), or cytokine release. These data argue against a role of early plasmin generation in the subsequent activation of other inflammatory pathways during human endotoxemia.

Introduction

LPS, present in the outer membrane of gram-negative bacteria, plays a pivotal role in triggering inflammatory responses during gram-negative sepsis. The human endotoxemia model, in which a bolus dose of LPS is administrated intravenously to healthy subjects, has been frequently used to study the mechanisms by which inflammatory systems are activated in man in vivo. Intravenous injection of LPS into healthy humans is associated with activation of the fibrinolytic system, the coagulation cascade, neutrophilic granulocytes, endothelial cells and the cytokine network. In particular activation of fibrinolysis is a very early phenomenon in the human response to LPS administration. Within two hours a marked increase in tissue-type plasminogen activator (tPA) can be detected, which leads to the generation of plasmin as reflected by an increase in plasmin-α2-antiplasmin (PAP) complexes. This activation is rapidly followed by an increase in plasminogen activator inhibitor-1 (PAI-1) levels, inhibiting the fibrinolytic system. These fibrinolytic changes precede and occur independently from activation of the coagulation system in this model.

Recent evidence indicates that the fibrinolytic system likely has functions different from its classical fibrin dissolving properties. Binding of plasmin(ogen) to surfaces plays a pivotal role in regulating the function of this system. Besides binding to fibrin, plasmin(ogen) can bind to many cell types, including neutrophilic granulocytes, monocytes, lymphocytes, platelets and endothelial cells. Upon binding to cells, conversion of plasminogen to plasmin is facilitated and cell-bound plasmin is protected from inactivation by α2-antiplasmin. Although the biological
function of cell-bound plasmin has been regarded mainly in terms of fibrinolytic activity, in recent years, it has become clear that plasmin can affect various cell functions. Cell-associated plasmin is considered to play an important role in extracellular matrix degradation and tissue remodeling. Interestingly, plasmin can also induce pro-inflammatory responses independent of its proteolytic properties. In vitro, plasmin was demonstrated to stimulate the release of cytokines and other inflammatory mediators by different cell types. Furthermore, plasmin induced cell adhesion and migration in vitro, and studies using plasminogen-deficient mice have provided evidence for an essential role of the plasminogen system in cell migration towards inflammatory sites. Moreover, plasmin can activate the p38 mitogen-activated protein kinase (MAPK) signaling pathway in monocytes, and activation of this pathway was recently shown to be of key importance for the inflammatory response to LPS in humans. Together, these findings implicate plasmin as a mediator of several cellular inflammatory responses. However, at present, the role of plasmin in systemic inflammation is unknown.

Tranexamic acid (Cyklokapron®) is a synthetic anti-fibrinolytic substance, which acts by competitively blocking the lysine binding sites of plasmin(ogen), thereby preventing binding to fibrin or cells. In vitro, tranexamic acid potently inhibited plasmin-induced proinflammatory responses.

The fact that the formation of plasmin is one of the earliest events after intravenous administration of LPS, together with the recent findings that plasmin is able to induce several cellular proinflammatory responses, including activation of p38 MAPK, led us to hypothesize that plasmin may play a role in the induction of LPS-induced inflammatory pathways. To test this hypothesis, we studied the effect of tranexamic acid infusion on activation of coagulation, granulocytes, endothelial cells and the cytokine network in healthy humans injected with a single dose of LPS.

Material and methods

Study design

Sixteen healthy men (19-34 years) were studied. The study was approved by the institutional scientific and ethics committees and written informed consent was obtained from all volunteers. Tranexamic acid (Cyklokapron®; Pharmacia & Upjohn, Woerden, the Netherlands) (2 gram in 100 mL sterile NaCl 0.9%; N = 8) or placebo (100 mL 0.9% sterile NaCl; N = 8) was administered intravenously over 30 minutes directly prior to LPS injection. All participants received a bolus intravenous injection of LPS (Escherichia coli lipopolysaccharide, lot G; US Pharmacopeia, Rockville, MD) at a dose of 4 ng/kg. Oral temperature, blood pressure, heart rate, and oxygen saturation were measured at half-hour intervals (Dinamap1846 SX; Critikon, Tampa, FL). Clinical symptoms such as headache, chills, nausea and myalgia were recorded throughout the study periods using a graded scale (0, absent; 1, weak; 2, moderate; 3, severe).
Blood collection

Blood was obtained before and at the end of the infusion of tranexamic acid or placebo (t = -0.5 h and t = 0 h), and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8 and 23 h after LPS injection. All blood samples, except those for the determination of leukocyte counts and differentials, were centrifuged at 3000 rpm for 15 minutes at 4°C, and plasma was stored at -20°C until assays were performed. Blood for FACS analysis was obtained directly before tranexamic acid or placebo infusion (t = -0.5 h) and at 1, 4, 6, and 23 h after LPS administration and put on ice.

Assays

Coagulation and fibrinolysis assays were done using citrated plasma, all other assays using EDTA plasma. The following ELISA’s were performed according to the instructions of the manufacturer and/or as described previously: D-dimer, F1+2 prothrombin fragment and thrombin-antithrombin (TAT) complexes (all Dade Behring, Marburg, Germany), tissue-type plasminogen activator (tPA) (Asserachrom tPA, Diagnostics Stago, Asnieres-sur-Seine, France), plasminogen activator inhibitor type 1 (PAI-1) (Monozyme, Charlottelund, Denmark), Elastase-a1-antitrypsin complex concentrations were measured with an ELISA modified from a previously described RIA procedure (28), bactericidal permeability-increasing protein (BPI) (29), von Willebrand factor (Dako, Glostrup, Denmark), soluble E-selectin (Diaclone, Fleming, France), tumor necrosis factor (TNF)-α, IL-6, IL-10 and IL-8 (all Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). Plasmin-a2-antiplasmin (PAP) complexes were measured by RIA as described previously. (30) Leukocyte counts and differentials were assessed by a STKR Coulter counter (Coulter, Bedfordshire, U.K.).

Flow Cytometry

Erythrocytes were lysed with ice-cold isotonic NH₄Cl solution (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA [pH 7.4]) for 10 minutes. The cells were centrifuged at 250 × g for 10 minutes at 4°C. The remaining cells were washed twice with FACS buffer (phosphate-buffered saline supplemented with 0.5% bovine serum albumin [BSA], 0.01% NaN₃, and 0.35 mM EDTA) and brought to a concentration of 4 × 10⁶ cells/mL in FACS buffer. All procedures were performed at 4°C. All FACS reagents were titrated to obtain optimal results, as recommended by the manufacturers. For each test at least 10⁶ cells were analyzed using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA). Cell surface staining was performed using the following anti-human mAbs: FITC-labeled mouse anti-human CD66b (clone 80H3; Immunotech, Marseille, France), PE-labeled mouse anti-human L-selectin (clone DREG-56), and APC-labeled mouse anti-human CD11b (clone ICRF44) (all BD Pharmingen, San Diego, CA). To correct for nonspecific staining, all analyses were also conducted with the appropriate isotype control Abs (FITC-, PE- and APC-
labeled murine IgG1, BD Pharmingen). Granulocytes were identified by forward and side-angle light scatter gating. Data are presented as the difference between mean fluorescence intensities (MFI) of specifically and nonspecifically stained cells.

**Statistical analysis**

All values are given as means ± SEM. Differences in time and between treatment groups were analyzed by mixed models analysis using SPSS for Windows (SPSS 11.5, Chicago, IL). A value of $P < 0.05$ was considered to represent a statistically significant difference.

**Results**

**Clinical features**

Intravenous injection of LPS elicited a febrile response, peaking after 4 h (38.4 ± 0.3 °C), together with tachycardia and transient flu-like symptoms, including headache, chills, nausea and myalgia. Infusion of tranexamic acid did not influence LPS-induced signs and symptoms, and no adverse events attributable to tranexamic acid infusion were observed (data not shown).

**Figure 1.** Tranexamic acid inhibits LPS-induced plasmin generation and activity. Plasma concentrations of D-dimer and PAP complexes after LPS administration (4 ng/kg IV, $t = 0$ h), preceded by a 30-minute infusion of placebo (○) or tranexamic acid (2 g IV, ●). Data are means ± SEM. $P$ value indicates the difference between treatment groups.
Inhibition of plasmin activity by tranexamic acid

Tranexamic acid competitively binds to the high affinity lysine binding sites on plasmin(ogen), thereby preventing direct action of plasmin on fibrin and cells and the surface-facilitated conversion of plasminogen to plasmin. To obtain evidence for the in vivo plasmin inhibitory activity of tranexamic acid during endotoxemia, we determined the plasma concentrations of D-dimer, a split product cleaved off from cross-linked fibrin by a direct action of plasmin, as a measure for plasmin activity and the plasma levels of PAP complexes, as a measure for plasmin generation (Fig. 1). LPS administration resulted in a profound rise in D-dimer, which reached a plateau phase from 3 h and peaked at 8 h (3400 ± 1000 μg/L), and a transient rise in PAP complexes, peaking after 1.5 h (276 ± 57 nmol/L). Tranexamic acid essentially prevented the increase in D-dimer levels (8 h: 1554 ± 388 μg/L; $P < 0.01$ vs placebo) and blunted the rise in PAP complexes (peak at 2 h: 172 ± 46 nmol/L; $P < 0.01$ vs placebo). Hence, these data indicate that tranexamic acid effectively inhibited plasmin generation and activity.

Activation and inhibition of fibrinolysis

LPS injection resulted in an early stimulation of the fibrinolytic system (Fig. 2), measured by a rise in tPA levels, peaking after 3 h ($P < 0.001$ vs baseline). This increase in tPA levels was followed by the secretion of its inhibitor PAI-1, peaking after 4 h ($P < 0.001$ vs baseline). Consistent with its mode of action, tranexamic acid did not influence the LPS-induced rises in tPA and PAI-1 concentrations.
Figure 3. Activation of coagulation.
Plasma concentrations of F1 + 2 and TAT complexes after LPS administration (4 ng/kg IV, \( t = 0 \) h), preceded by a 30-minute infusion of placebo (○) or tranexamic acid (2 g IV, •). Data are means ± SEM. \( P \) values (NS, not significant) indicate the difference between treatment groups.

 Activation of the coagulation system.
LPS administration was associated with thrombin generation, as reflected by increases in the plasma levels of the prothrombin fragment F1 + 2 and TAT complexes (both \( P < 0.001 \) vs baseline). Tranexamic acid did not affect the LPS-induced thrombin generation (Fig. 3).

 Leukocyte activation
LPS injection induced activation of neutrophilic granulocytes, as reflected by a biphasic change in neutrophil counts involving an initial decrease with a nadir at 1 hour followed by neutrophilia peaking at 8 h (\( P < 0.001 \) vs baseline; Fig. 4). Furthermore, LPS administration induced an up-regulation of the activation markers CD11b (Fig. 4) and CD66b (data not shown) at the surface of circulating granulocytes with a concurrent down-modulation of L-selectin (Fig. 4) (all \( P < .01 \) vs baseline). Moreover, LPS injection resulted in an increase in degranulation products as measured by BPI (Fig. 4) and elastase-α1-antitrypsin complexes (data not shown) (both \( P < 0.001 \) vs baseline). Tranexamic acid did not modify any of these LPS-induced changes.
**Endothelial cell response**

LPS administration elicited endothelial cell activation, as indicated by profound increases in the plasma concentrations of vWF and soluble E-selectin (both $P < 0.001$ vs baseline). Tranexamic acid infusion did not alter these LPS-induced endothelial cell responses (Fig. 5).

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*Figure 4. Activation of neutrophils.* Circulating neutrophil counts, mean fluorescence intensity (MFI) of CD11b and L-selectin on granulocytes and the degranulation product BPI after LPS administration (4 ng/kg IV, $t = 0$ h), preceded by a 30-minute infusion of placebo (○) or tranexamic acid (2 g IV, ●). Data are means ± SEM. FACS data (CD11b and L-selectin) are expressed as the difference between specific MFI and nonspecific MFI. $P$ values (NS, not significant) indicate the difference between treatment groups.

*Figure 5. Endothelial cell activation.* Plasma concentrations of vWF and soluble E-selectin after LPS administration (4 ng/kg IV, $t = 0$ h), preceded by a 30-minute infusion of placebo (○) or tranexamic acid (2 g IV, ●). Data are means ± SEM. $P$ values (NS, not significant) indicate the difference between treatment groups.
Cytokine response

LPS injection was associated with a transient rise in the plasma concentration of TNF, IL-6, IL-8 and IL-10, peaking after 2-3 h (all $P < 0.001$ vs baseline). Tranexamic acid infusion did not influence these LPS-induced cytokine responses (Fig. 6).

![Graphs showing cytokine response](image)

Figure 6. Cytokine response. Plasma levels of TNF-α, IL-6, IL-10 and IL-8 after LPS administration (4 ng/kg IV, $t=0$ h), preceded by a 30-minute infusion of placebo (○) or tranexamic acid (2 g IV, ●). Data are means ± SEM. $P$ values (NS, not significant) indicate the difference between treatment groups.

Discussion

In recent years it has become clear that plasmin has functions beyond its classical proteolytic and fibrin degrading properties. *In vitro* and animal studies have provided evidence for a stimulatory effect of plasmin on cellular proinflammatory responses. The current investigation is the first to examine the role of plasmin during a systemic inflammatory response in humans *in vivo*. We here demonstrate that although pre-treatment with tranexamic acid strongly inhibited LPS-induced plasmin activation, it did not influence sensitive markers of the activation of proinflammatory pathways that accompany endotoxemia, including effects on the coagulation cascade, granulocytes, endothelial cells and the cytokine network. These findings suggest that plasmin is not involved in the induction of systemic inflammatory responses during human endotoxemia.
Binding of plasmin(ogen) to fibrin or cell surfaces is of crucial importance in regulating its function. Plasmin(ogen) binds to fibrin and cells via its lysine binding sites, which are associated with its kringle domains and recognize carboxy-terminal lysines of surface proteins. Upon binding plasmin activity is increased and the conversion of plasminogen to plasmin is facilitated. Tranexamic acid competitively binds to the lysine binding sites of plasmin(ogen), thereby blocking the binding to fibrin and cells. Although plasmin can still be formed under these circumstances, its activity and the surface-facilitated plasmin generation are inhibited. In line with this mode of action, we found that pre-treatment with tranexamic acid strongly reduced the LPS-induced rise in the plasma levels of D-dimer, a split product cleaved off from cross-linked fibrin by a direct action of plasmin, providing direct evidence for the virtually complete inhibition of plasmin activity on fibrin in vivo. Furthermore, pre-treatment with tranexamic acid decreased the levels of circulating PAP complexes, indicating that apart from the strong inhibition of plasmin activity, plasmin generation was also inhibited by tranexamic acid, albeit to a lesser extent. As expected, pre-treatment with tranexamic acid did not influence the LPS-induced increase in tPA levels, nor did it change the subsequent rise in PAI-1 concentration.

LPS injection induced thrombin generation, as shown by a rise in the plasma concentrations of F1+2 and TAT complexes, which indicates stimulation of the coagulation cascade. Tissue factor plays a pivotal role herein. Indeed, LPS administration to healthy humans resulted in a marked increase in monocytic tissue factor mRNA expression, and treatment with recombinant tissue factor pathway inhibitor strongly inhibited the associated coagulation activation. Interestingly, plasmin is able to induce tissue factor expression on monocytes in vitro, which was inhibited by tranexamic acid. This finding led us to investigate the effect of tranexamic acid on coagulation activation after LPS injection. However, we did not find any influence of inhibition of plasmin activity by tranexamic acid on activation of the coagulation cascade. Of note, earlier investigations have demonstrated that the fibrinolytic changes during endotoxemia are completely independent of coagulation activation. In the present study we show, in turn, that the activation of the coagulation system occurs independent of plasmin activation.

Neutrophilic granulocytes are activated upon infection or inflammation and have been implicated in the pathogenesis of tissue injury during severe sepsis. Plasmin induced neutrophil aggregation and increased neutrophil adhesion to endothelial cells in vitro, an effect that could be inhibited by tranexamic acid. The plasmin-induced neutrophil adherence was mediated through an upregulation of CD18 neutrophil cell surface glycoprotein, reflecting neutrophil activation. These data suggest that plasmin is able to activate neutrophils, which can be abrogated by tranexamic acid. To investigate neutrophil activation we measured CD11b, CD66b, L-selectin and circulating neutrophilic degranulation products. CD11b and CD66b expression on neutrophils are both upregulated after LPS infusion. CD66b is a glycoprotein believed to be involved in neutrophil activation and migration, by means of regulating the adhesive activity of CD11b/CD18. L-selectin that is constitutively
present on the neutrophil membrane is necessary for initial neutrophil-endothelial cell interaction that results in rolling of neutrophils on endothelium \(^{36}\). Activation of neutrophils by LPS causes shedding of L-selectin \(^{24}\). In contrast to the \textit{in vitro} data, our findings show that infusion of tranexamic acid does not influence the neutrophilic responses to LPS administration in humans \textit{in vivo} and has no effect on neutrophil activation, as reflected by unaltered upregulation of CD11b and CD66b, downmodulation of L-selectin and rise in circulating neutrophilic degranulation products.

Endothelial cells play a pivotal role in the inflammatory response to systemic infection \(^{37,38}\). Plasmin can influence endothelial cell behavior \textit{in vitro}. Endothelial cells incubated with plasmin showed an enhanced release of arachidonate, the precursor of leukotriene B4 (LTB4) and other eicosanoids \(^{16}\), which was inhibited by tranexamic acid. Furthermore, plasmin induced endothelial cell retraction evidenced by loss of cell-cell contacts and increased permeability \(^{17}\), and stimulated endothelial cell migration \textit{in vitro} \(^{18}\). Together these data implicate plasmin as a mediator of endothelial cell activation. However, in the present study, inhibition of LPS-induced plasmin activity did not affect the endothelial cell activation, measured by plasma levels of von Willebrand factor and soluble E-selectin.

The release of cytokines into the circulation is a characteristic feature of endotoxemia, predominantly mediated by monocytes and macrophages. Stimulation of human peripheral monocytes with plasmin \textit{in vitro} induced an up-regulation of several inflammatory mediators, including TNF-\(\alpha\), IL-1\(\alpha\), IL-1\(\beta\), monocyte chemoattractant protein (MCP)-1 and LTB4 \(^{13,15}\). Tranexamic acid attenuated cytokine mRNA expression elicited by plasmin \(^{13}\). Plasmin-induced expression of TNF-\(\alpha\), IL-1\(\alpha\) and IL-1\(\beta\) involved AP-1 and NF-\(\kappa\)B activation \(^{13}\), whereas plasmin-induced monocyte expression of MCP-1 and CD40 was triggered via activation of the p38 MAPK and Janus Kinase/STAT signaling pathways \(^{14}\). Syrovets et al. demonstrated that ciglitazone inhibited cytokine release from plasmin-stimulated monocytes by inhibition of AP-1 and NF-\(\kappa\)B activation via modulation of p38 MAPK activity \(^{23}\). In accordance, a specific p38 MAPK inhibitor significantly diminished proinflammatory gene expression by plasmin-stimulated peripheral monocytes \(^{23,39}\). Together, these data indicate that plasmin induces monocytic cytokine production at least in part via p38 MAPK activation. Recently, our laboratory demonstrated that the p38 MAPK signaling pathway is important for induction of the inflammatory response to LPS in humans. Indeed, intravenous injection of LPS resulted in a transient activation of p38 MAPK \(^{24}\), and more importantly, a specific p38 MAPK inhibitor strongly inhibited the LPS-induced cytokine production and other proinflammatory responses in humans \textit{in vivo} \(^{24,25}\). In spite of this abundant \textit{in vitro} evidence that plasmin can induce p38 MAPK activation and cytokine production, inhibition of plasmin activity by tranexamic acid did not affect the cytokine response in the present study. It should be noted that we did not measure p38 MAPK activation in blood cells in the current study, and thus we can only speculate on the effects of tranexamic acid on p38 MAPK.
activation in our study subjects. Indeed, in a more general way, it remains to be
established whether plasmin can activate p38 MAPK in vivo.

In vitro and animal studies have indicated that plasmin can activate various
inflammatory pathways implicated in the host response to endotoxemia. We here
demonstrate that although active plasmin is generated early after intravenous injection
of LPS into normal subjects, it does not contribute to a significant extent to activation
of the coagulation system, granulocytes, the vascular endothelium or the cytokine
network. By the nature of our experiment, performed in healthy human beings, we
cannot exclude that plasmin does play a role in endotoxemia or infection models in
which more severe challenges are given. Investigations in animals are warranted to
determine the potential role of plasmin in a lethal systemic inflammatory response
syndrome.

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