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Plasmin-dependent proteolysis of Tissue Factor Pathway Inhibitor in a mouse model of endotoxemia

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Summary

Background—Development of a procoagulant state in sepsis, due to aberrant expression of tissue factor (TF) and sharp decrease of its major inhibitor tissue factor pathway inhibitor (TFPI), could lead to microthrombotic organ failure. The mechanism for the decline of TFPI activity in the lung could involve plasmin-mediated cleavage of the inhibitor.

Objective—To investigate the effect of plasmin generation on lung-associated TFPI activity, in normal conditions and during infusion of endotoxin (LPS) in mice.

Methods—Plasmin generation and TFPI activity were assayed in the lungs of mice deficient of tissue-type plasminogen activator (t-PA) or plasminogen (Plg), at 2-hrs after LPS or saline injection.

Results—The sharp loss of lung-associated TFPI activity at 2-hrs post LPS paralleled the abrupt increase of plasmin generation. TFPI activity was significantly retained in both t-PA-/- and Plg-/- mice, which are unable to generate plasmin.

Conclusion—The increased plasmin generation during the early stages of sepsis could cleave/inactivate TFPI and thus lead to thrombotic complications.

Keywords

endotoxin; lung; plasminogen activating system deficient mice; plasmin; tissue factor pathway inhibitor

Introduction

Although the pathogenesis of septic acute respiratory distress syndrome is not precisely understood, inflammation, coagulation and apoptosis are intrinsically linked in sepsis[1]. Endotoxin (LPS) exposure causes development of a procoagulant state, probably due to the fact that the endothelium becomes more thrombogenic instead of thromboresistant.

Activation of tissue factor (TF)-dependent coagulation leads to thrombin formation and subsequent fibrin deposition in many organs[2, 3]. Aberrant expression of TF may cause

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thrombosis in various clinical disorders, including sepsis. LPS-challenged endothelial cells (EC) and monocytes express TF[4, 5].

Tissue Factor Pathway Inhibitor (TFPI) is a major inhibitor of the TF-FVIIa-initiated coagulation *in vivo*. Others and we suggested that during early sepsis, the available TFPI might not adequately balance the increased TF-dependent coagulation activation[6, 7]. Our previous work in the baboon model of sepsis indicated that almost 90% of TFPI inhibitory activity in the lung was lost during the first two hours post *E. coli* challenge. This loss could influence the vascular and perivascular fibrin deposition leading to microthrombotic organ failure. We also found that the early decrease of lung-associated TFPI activity coincided with the release of tissue-type plasminogen activator (tPA) from the tissue and the consequent peak of plasmin generation. Based on this temporal correlation, we suggested that plasmin might be partly responsible for proteolytic degradation of TFPI in the early stages of sepsis[6].

Plasmin was shown to cleave TFPI without leaving degradation products[8], a process that was proposed to contribute to the decrease of endothelial- and monocyte-associated TFPI in various conditions[9-11]. Nevertheless, other proteolytic enzymes, including neutrophil elastases[12, 13] and bacterial proteases [14], could also degrade TFPI during the early stages of sepsis.

Here we addressed directly the effect of plasmin generation on lung- associated TFPI activity by developing a mouse model of endotoxemia where we injected LPS and assayed thrombin and plasmin generation and TFPI activity after 2-hrs. Using mice deficient of plasminogen activating (PA) system components, namely tPA and plasminogen (Plg), helped us understand the role of individual pathways and allowed us to conclude that plasmin generation could be at least in part responsible for the observed early loss of TFPI-mediated anticoagulant activity in the lung.

Materials and Methods

Mouse model of endotoxemia

The genetic background for the tPA-/- and Plg-/- mice and their corresponding wild-type (WT) counterparts was 75% C57Bl/6 : 25% 129svJ.

All mice were generated in the KU Leuven animal facility. Mice were housed at 22°C on 12 hrs light/dark cycle, with free access to chow and water.

9-10 weeks- old mice of both genders (20-30 g body-weight; 5-6 animals per group) were intra-peritoneally (*i.p.*) injected with either LPS (E. coli 0111:B4 [Sigma-Aldrich, cat # L2630]; 40 mg/kg from a stock of 5 mg LPS/mL in sterile saline) or saline only. Mice were sacrificed before and 2-hrs after the LPS/saline injection, by *i.p.* injection of 65 mg/kg Nembutal (Abbott Laboratories, North Chicago, IL). Blood was collected on citric acid-sodium citrate (1.5% citric acid and 2.5% tri-sodium citrate) via the retro-orbital sinus and plasma was stored at –80°C. Animals were perfused by transcardiac perfusion for 10-15 min with sterile saline solution, after which lungs were removed and weighed, and portions were snap-frozen in liquid nitrogen for protein and RNA extraction, and OCT-embedded for fibrin overlay.

All animal experiments were approved by the local ethical committee (KU Leuven, Belgium) and performed in accordance with the NIH Guide for Care and Use of Laboratory Animals[15] and the guiding principles of the ISTH.

Quantitative real time (qRT)-PCR

qRT-PCR was used to measure TFPI-α, TFPI-β, TF, tPA, PAI-1 and β-actin gene expression[6]. Lungs were homogenized in TRIzol (Invitrogen) according to the manufacturer. Primers were designed using Primer Express software (Applied Biosystems, Foster City, CA). For each sample, 2 μg of total RNA was reverse-transcribed using Omniscript Reverse Transcription System (Qiagen, Valencia, CA) with oligo-dT primers. qRT-PCR was performed in duplicate with 1 μL of the 20-μL reverse transcription reaction products by using iTaq SYBR Green Supermix with ROX kit (Bio-Rad, Hercules, CA) in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Relative quantification of gene expression with the standard curve method was performed following the manufacturer's protocol (Applied Biosystems). The relative expression of target genes was normalized with β-actin mRNA level as housekeeping gene.

Preparation of lung extracts

Extracts were prepared by lysis of lungs on ice in 10 mM phosphate buffer, pH 7.2, containing 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100 and 0.2% sodium azide. Homogenates were centrifuged at 14,500 × g for 15 min at 4°C, and the supernatants representing the lung lysates were stored at –80°C.

TFPI and TF Activity

Lung homogenates were dialyzed for 24-hrs against several changes of 50 mmol/L Tris-HCl buffer, pH 7.4, to remove the detergents[6]. TFPI activity was measured by evaluating the ability of TFPI to inhibit the activation of FX by TF-FVIIa[6, 16]. TFPI activity was extrapolated from a standard curve constructed with serial dilutions of pooled normal mouse plasma, and expressed as mUnits/mg total protein.

TF activity was measured through a one-stage clotting assay (http://tf7.org/mlabprotocols.htm).

In situ zymography on fibrin overlay

The assay was performed essentially as described[17]. Cryostat sections (7-μm thick) of unfixed tissue were air-dried for 30 min, a fibrin overlay was added at 37°C, a coverslip was mounted over the section and incubated for 30 min at RT to polymerize the overlay. The fibrin overlay was prepared by clotting human fibrinogen with human thrombin, by mixing at 47°C human fibrinogen (4 mg/mL f.c.), human plasminogen (0.1 μmol/L f.c.) and low melting-point agarose (Ultra pure electrophoresis grade, Gibco; 0.5% f.c.) in 50 mmol/L Tris buffer pH 7.4 containing 10 mmol/L $CaCl_2$ and 0.05% Brij, with human thrombin (2.8 nmol/L f.c.). Slides were incubated horizontally in a humidified chamber for 1 hour at 37°C for assessment of total PA activity. Lysis of the fibrin overlay was monitored under a dark-field microscope and appeared as clear zones in the opaque film. Overlays were analyzed by computer-assisted image analysis using ImageJ 1.42b software (NIH). Lysis is expressed as a ratio between the lysis area *vs.* the total section areas (μm^2).

Other assays

Total protein was assayed by BCA (Pierce). tPA and PAI-1 antigen levels were measured with specific ELISAs[18], using home-made murine monoclonal antibodies against m-PAI-1 (H34G6D10) and m-tPA (H27B2) for coating and biotinylated rabbit anti-mouse IgG secondary antibodies for detection. Commercial ELISA kits were used to measure plasma levels of: thrombin-antithrombin (TAT) (Enzygnost, Dade Behring) and plasmin-α_2-antiplasmin (PAP) (BioTang Inc.), D-Dimer (ASSERACHROM D-Di Diagnostica Stago Inc.), and TNF-α and IL-1 (Cytokine 10-plex for Luminex, Biosource).

Statistical analysis

Data are shown as mean ± SEM for the number of animals studied. We used Prism version 5.0c (GraphPad) to perform the unpaired *t* test and 1-way analysis of variance (ANOVA). Values of $P < 0.05$ were considered statistically significant. Assays were typically run in duplicate.

Results

Effect of LPS on TFPI activity in the lungs of mice deficient of tPA and Plg

TFPI activity, measured as the ability of TFPI to inhibit the activation of FX by TF-FVIIa, was significantly higher (~ 2-fold) in control lungs from both tPA-/- and Plg-/- mice, as compared to values obtained for the corresponding WT animals (Fig. 1, a and b). This indicates that more TFPI activity is being retained in the absence of plasmin generation.

TFPI activity in lung extracts decreased by ~5-fold after LPS challenge in WT mice, and by ~2-fold in both tPA-/- and Plg-/- mice, in comparison with the control mice within each experimental group (Fig. 1). Thus, TFPI activity in the lungs from PA system deficient mice challenged with LPS was ~4-fold higher than in the corresponding WT mice (Fig. 1, a and b).

The apparent ~20% difference in basal TFPI activity between controls in the WT groups from tPA-/- and Plg-/- mice was not statistically significant. The range of individual values was 100-300 mU TFPI/mg total protein for both WT groups (n=6) (Fig. 1).

LPS challenge induces a rapid burst of plasmin generation in the lung

In situ zymography on lung section fibrin overlays shows that in WT mice, LPS-challenge significantly decreased the plasmin generation as compared with control mice (Fig. 2a). This assay primarily detects tPA activity[17]. Addition of neutralizing antibodies anti-tPA completely inhibited the lysis, demonstrating that the observed fibrin lysis was fully dependent on t-PA (not shown).

This decrease may reflect an LPS- induced acute release of tPA from endothelial storage pools, which presumably led to a burst of plasmin generation, as illustrated by the ~ 5-fold increase in the plasma concentration of PAP complexes (Fig. 2b, note the interrupted Y-axis), and confirmed by the higher levels of D-dimer (see also Fig. 3b). Accordingly, we found that the elevated plasmin coincides with similarly increased thrombin generation measured by the TAT assay (Fig. 2c, WT). The high amount of plasmin generation is probably due to the increase of tPA release from the lung endothelial stores in response to LPS-induced thrombin generation, as shown by zymography data (Fig. 2a), as well as to a slight increase of tPA antigen in the lungs induced by LPS (Fig. 2d). The fact that the zymography shows less lysis reflects both an increased release of tPA from tissue stores and the inhibition of tPA by PAI-1, which is also strongly induced by LPS (Fig. 2e).

Both zymography and PAP assays demonstrated that, as expected, the lungs of tPA-/- and Plg -/- mice generated very little plasmin regardless of the experimental condition (Fig. 2, a and b; compare C *vs.* LPS for tPA-/- and Plg-/- groups; note the interruption of Y-axis in panel b).

LPS-induced thrombin generation occurred strongly for the WT, but was lower in tPA-/- and Plg-/- mice, which could reflect the higher TFPI activity in these mice (Fig. 2c).

Coagulation and cytokine responses

Administration of LPS to WT mice resulted in a strong prothrombotic response, characterized by increased TF activity associated with the lung tissue (Fig. 3a), which correlates with thrombin generation, as reflected by increased plasma levels of TAT complexes and D-dimer (Figs. 2c and 3b).

Activation of coagulation was accompanied by concurrent inhibition of anticoagulant mechanisms, such as decreased levels of protein C (not shown) and increased soluble TM levels (Fig. 3c).

LPS injection was also associated with a significant rise in the plasma concentration of the pro-inflammatory cytokines TNF-α and IL-1 (Fig. 3, d and e).

Effects of LPS on TFPI, TF, tPA and PAI-1 mRNA expression in the lungs of mice with plasminogen activation system deficiencies

mRNA expression for both TFPI-α and TFPI-β decreased ~2-fold in WT mice after 2-hrs challenge with LPS as compared with control animals (Fig. 4, a and b), whereas TF mRNA similarly increased (Fig. 4c). tPA and PAI-1 mRNA expression was significantly increased after LPS (Fig. 4d).

In tPA-/- mice the mRNA expression of TFPI-α and TFPI-β did not change significantly vs. WT, in neither control nor LPS- challenged mice, but it decreased at 2-hrs LPS vs. control, following the same trend as WT mice (Fig. 4, a and b). TF expression did not change in tPA-/- control mice either, but increased significantly after 2-hrs LPS in comparison with both LPS- treated WT mice and tPA-/- controls (Fig. 4c).

Discussion

The pathology of sepsis results, in part, from a complex deregulation of normal haemostasis leading to activation of the procoagulant pathways concurrently with impairment of both anticoagulant and fibrinolytic systems[19].

Our previous work in non-human primate sepsis models[6] showed that TF was significantly up-regulated at 2-hrs after infusion of *E. coli* into baboons, a time-point that matches the peak plasma levels of LPS, TNF-α and other inflammatory cytokines and coincides with the early stage of the procoagulant response, dominated by thrombin and plasmin production, as well as sharply decreased TFPI activity. We also observed an inverse temporal correlation between plasmin generation and the decrease of TFPI activity, with maximal effects at 2-hrs post challenge and reversal to basal levels by 8-hrs for both parameters. The burst of plasmin generation at 2-hrs and decrease to basal levels by 3-4 hrs post LPS was also documented for human experimental endotoxemia[20, 21]. Based on these data, we hypothesized that plasmin could be responsible for the proteolytic degradation of TFPI. Since other proteases produced by the host[12, 13] or the pathogen, such as omptins[14] could also degrade TFPI, here we used genetic mouse models and LPS-challenge instead of live *E. coli*, to investigate the direct contribution of plasmin to TFPI degradation during the early stages of endotoxemia, and to exclude the possible effects of bacterial proteases on TFPI. Similarly to bacterial challenge in baboons, mice exposed for 2-hrs to LPS showed increased inflammatory responses (TNF-α, IL-1), activation of procoagulant pathways (increased TF, TAT, D-dimer, sTM), and deregulated fibrinolysis. Like in other models of *E. coli* or LPS challenge in baboons[22] or in humans[23], the increased plasmin generation, as indicated by the higher levels of plasma PAPc, is coincidental with thrombin formation, shown by plasma TAT levels, and reflects the burst of plasmin production in the lung probably due to thrombin-induced release of t-PA[24]. Once released, t-PA rapidly activates

plasminogen to plasmin before is inactivated by the inhibitor PAI-1, which is strongly induced in sepsis. This assumption is supported by the decrease in active t-PA observed by zymography in the lung sections of LPS challenged mice. Both t-PA and PAI-1 mRNA expression increased after LPS, albeit at a different extent.

TFPI activity measured in lung extracts was greatly affected by LPS injection, leading to ~ 5-times decreased anticoagulant activity in WT mice, which confirms and extends our similar observations made in baboons injected with *E. coli*[6]. Although this decrease could indicate a deregulated balance between TF and TFPI, it is unlikely that the increase of TF alone (~1.5-times) could be wholly responsible for the decreased TFPI activity. The reduced mRNA expression for both forms of TFPI (α and β) observed after LPS challenge could also partly contribute to the decreased TFPI activity.

In an attempt to verify that plasmin is indeed responsible for TFPI inactivation at short time after LPS administration, we tested the effects of tPA and Plg deficiency on TFPI activity. Plasminogen deficiency in mice is compatible with growth to adulthood, but the absence of this key fibrinolytic enzyme results in the development of spontaneous microvascular thrombosis, impaired tissue repair and diminished life expectancy[25].

We found that the functional activity of lung- associated TFPI was significantly higher in control tPA-/- and Plg-/- mice, as well as significantly retained after LPS-challenge in the PA system deficient mice, as compared with WT animals. Our results suggest that plasmin generation could be one significant factor responsible for the observed decrease of TFPI activity during the early stages of sepsis. The higher basal lung TFPI levels in the PA system KO mice could also indicate the presence of adaptive mechanisms developed to counteract decreased fibrinolysis and thus play a role in downstream events induced by LPS, such as coagulopathic responses.

The fact that TFPI activity still decreases in tPA-/- and Plg-/- mice after LPS challenge, albeit by ~2-fold only, indicates that plasmin-mediated degradation is not solely responsible for TFPI inactivation. Other mechanisms may include decreased TFPI mRNA expression, as illustrated by our data and documented by other groups[26, 27], as well as TFPI degradation by other proteolytic enzymes, such as neutrophil elastase[12, 13].

Our findings suggest that low and/or localized basal levels of plasmin could, to some extent, contribute to the normal turn-over of TFPI, but the abrupt increase of plasmin generation that occurs during the early stages of sepsis is significantly responsible for the dramatic decrease of TFPI activity. We suggest that alongside other proteases released during sepsis, plasmin can significantly contribute to the proteolytic degradation of TFPI, particularly during the early stages of endotoxemia, when there are still untapped stores of tPA, and the PAI-1 levels did not reach the peak yet.

Plasmin-dependent TFPI vulnerability may enhance the early coagulopathic response and contribute to sepsis DIC. Plasmin is able to cleave TFPI from endothelial cells[8, 11], and to decrease the surface-associated TFPI on circulating monocytes after thrombolytic therapy[10], which may lead to thrombotic complications after fibrinolysis in acute myocardial infarction[28], as well as to vascular and perivascular fibrin deposition[6]. This could contribute to fibrotic repair that may follow acute pleural injury[11], and to pulmonary intravascular coagulation and acute respiratory distress syndrome associated with sepsis.

Acknowledgments

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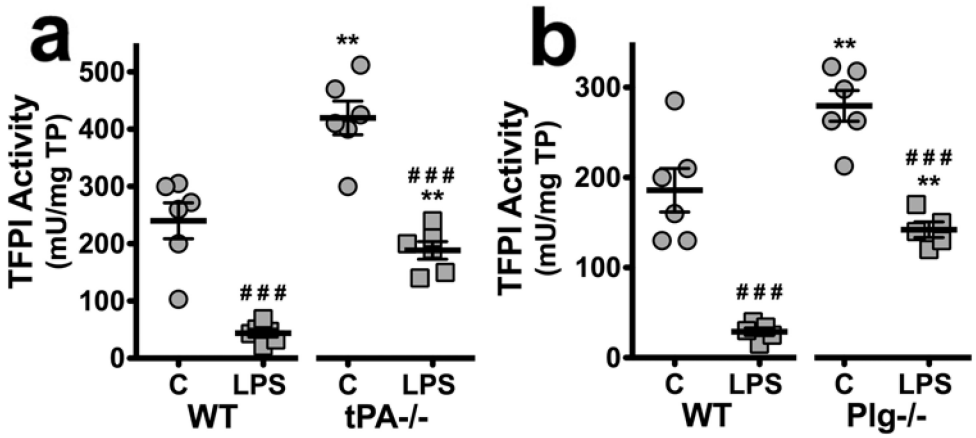

Figure 1. Lung- associated TFPI activity and effect of LPS in mice with plasminogen activating system deficiencies

TFPI activity was measured as the ability of TFPI to inhibit the activation of FX by preformed TF-FVIIa, in lung extracts from WT, tPA-/- (a) and Plg-/- (b) mice, in normal conditions and at 2-hrs after challenge with lipopolysaccharide (LPS). Data are scatterplots and mean ± SEM. For WT *vs.* tPA-/- and Plg-/-: $^{**}P < .001$ and $^{*}P < .01$. For LPS *vs.* Control: $^{\#\#\#}P < .0001$.

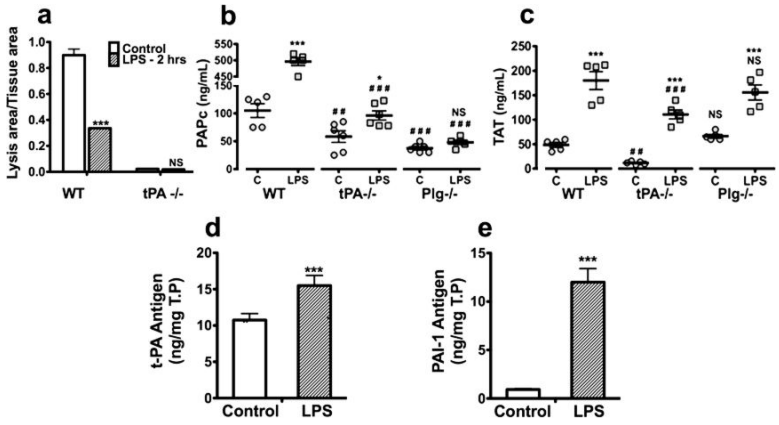

Figure 2. Effect of LPS on the fibrinolytic response and plasmin generation in the lung

(a) The activity of plasmin generated in the lungs of mice during the first 2-hrs after LPS challenge was assessed by measuring fibrin lysis areas on lung sections from WT and tPA-/- mice. (b) Scatterplot shows the levels of plasmin- α_2-antiplasmin complexes (PAPc) in the plasma were measured for all the genotypes tested at T0 (Control, C) and after 2-hrs following LPS challenge (LPS). (c) Scatterplot showing the concentration of thrombin-antithrombin (TAT) complexes in the plasma (same samples as for panel b). Levels of t-PA (d) and PAI-1 (e) were measured for control and LPS-injected WT mice. Data are mean ± SEM. For WT *vs.* tPA-/- and Plg-/-: $^{###}P < .0001$, $^{##}P < .001$ and NS: non-significant. For LPS *vs.* Control: $^{***}P < .0001$, $^{*}P < .01$ and N.S.: non-significant.

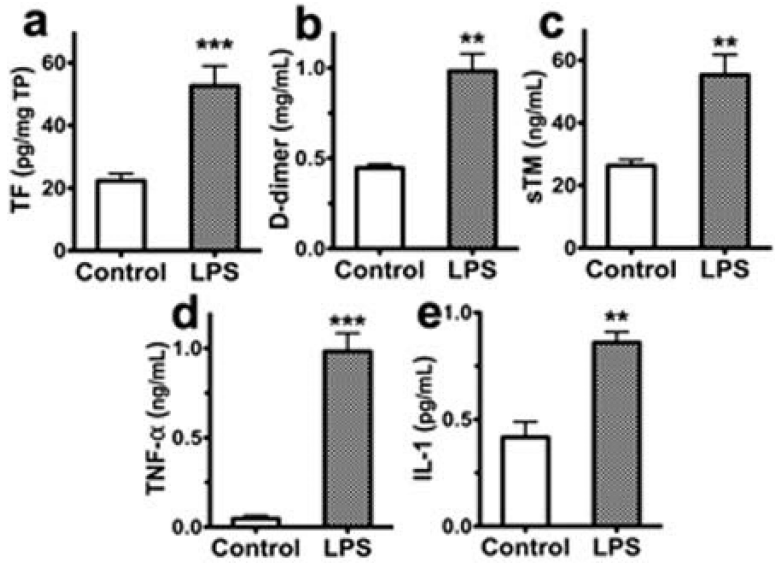

Figure 3. Effect of LPS on the activation of coagulation and inflammatory markers in the lung

TF activity (a), concentration of D-dimer (b), soluble thrombomodulin (sTM, c), TNF-a (d) and IL-1 (e) were measured in lung homogenates (a and c) or plasma (b, d and e) of WT mice 2-hrs post challenge. Data are mean ± SEM. LPS *vs.* Control: ***$P < .0001$ and **$P < .001$.

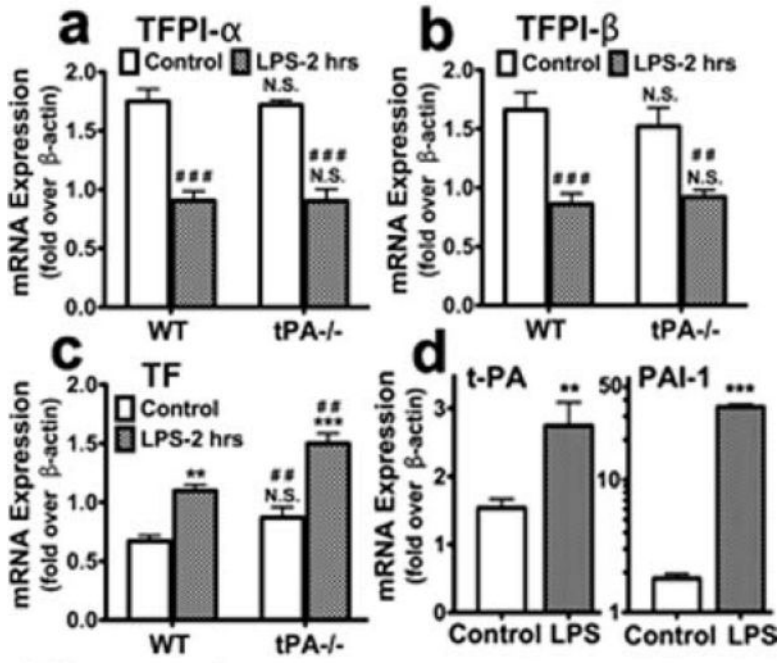

Figure 4. Effect of plasminogen activating system deficiencies on mRNA expression of TFPI, TF, tPA and PAI-1

mRNA levels were measured by qRT-PCR in the lungs of WT or tPA-/- mice, either Control or 2-hrs post challenge with LPS. Data are mean ± SEM. For (a – c): tPA-/- *vs.* WT $^{***}P < .0001$ and N.S: non-significant; and LPS *vs.* Control $^{###}P < .0001$ and $^{##}P < .001$. For d): LPS *vs.* Control $^{***}P < .0001$ and $^{**}P < .001$.