The Proteolytic Inactivation of Protein Z-Dependent Protease Inhibitor by Neutrophil Elastase Might Promote the Procoagulant Activity of Neutrophil Extracellular Traps in Sepsis

Elsa P Bianchini, Mahita Razanakolona, Julie Helms, Fouzia Zouiti, Amélie Couteau-Chardon, Viviana Marin-Esteban, Luc de Chaisemartin, Allan De-Carvalho, Roselyne Bironien, Sylvie Chollet-Martin, et al.

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Elsa P. Bianchini[1,§], Mahita Razanakolona[1], Julie Helms[2,3], Fouzia Zouiti[4], Amélie Couteau-Chardon[5], Viviana Marin-Esteban[6], Luc de Chaisemartin[6,7], Allan De-Carvalho[1], Roselyne Bironien[8], Sylvie Chollet-Martin[6,7], Cécile V. Denis[1], Jean-Luc Diehl[5,9], Marc Vasse[1,8], Ferhat Meziani[2,10], Delphine Borgel[1,11]

[1]HITh, UMR_S1176, Institut National de la Santé et de la Recherche Médicale, Université Paris-Saclay, Le Kremlin-Bicêtre, France.

[2]Université de Strasbourg, Service de Médecine Intensive-Réanimation, Nouvel Hôpital Civil, Hôpitaux universitaires de Strasbourg, Faculté de Médecine, Strasbourg, France.

[3]UMR_S1109, Institut National de la Santé et de la Recherche Médicale, Faculté de Médecine, Fédération Hospitalo-Universitaire, Fédération de Médecine Translationnelle de Strasbourg (FMTS), Université de Strasbourg, Strasbourg, France.

[4]Service d'Hématologie biologique, Hôpital Antoine Béclère, APHP, Université Paris-Saclay, Clamart, France.

[5]Service de Médecine Intensive-Réanimation, Hôpital Européen Georges Pompidou, APHP, Paris, France.

[6]UMR_996, Institut National de la Santé et de la Recherche Médicale, Université Paris-Saclay, Chatenay-Malabry, France.

[7]Laboratoire d'Immunologie, Hôpital Bichat, APHP, Paris, France.

[8]Service de Biologie Clinique, Hôpital Foch, Suresnes, France

[9]UMR_S1140, Institut National de la Santé et de la Recherche Médicale, Université de Paris, Paris, France.

[10]UMR_1260, Institut National de la Santé et de la Recherche Médicale, Fédération de Médecine Translationnelle de Strasbourg (FMTS), Strasbourg, France

[11]Laboratoire d'Hématologie Biologique, Hôpital Necker, APHP, Paris, France

[§]**Corresponding author**: Elsa P. Bianchini, PhD, INSERM U1176, 80 rue du General Leclerc, F-94276, Le Kremlin-Bicêtre, France. Tel: +33-149-595-646.

e-mail: elsa.bianchini@universite-paris-saclay.fr

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SUMMARY

Septic shock is the archetypal clinical setting in which extensive cross talk between inflammation and coagulation dysregulates the latter. The main anticoagulant systems are systematically impaired, depleted and/or downregulated. Protein Z-dependent protease inhibitor (ZPI) is an anticoagulant serpin that not only targets coagulation factors Xa and XIa but also acts as an acute phase reactant whose plasma concentration rises in inflammatory settings. The objective of the present study was to assess the plasma ZPI antigen level in a cohort of patients suffering from septic shock with or without overt-disseminated intravascular coagulation (DIC). The plasma ZPI antigen level was approximately 2.5-fold higher in the patient group (n=100; 38 with DIC and 62 without) than in healthy controls (n=31). The elevation's magnitude did not appear to depend on the presence/absence of DIC. Furthermore, Western blots revealed the presence of cleaved ZPI in plasma from patients with severe sepsis, independently of the DIC status. *In vitro*, ZPI was proteolytically inactivated by purified neutrophil elastase (NE) and by NE on the surface of neutrophil extracellular traps (NETs). The electrophoretic pattern of ZPI after NE-catalyzed proteolysis was very similar to that resulting from the clotting process - suggesting that the cleaved ZPI observed in severe sepsis plasma is devoid of anticoagulant activity. Taken as a whole, our results (i) suggest that NE is involved in ZPI inactivation during sepsis, and (ii) reveal a novel putative mechanism for the procoagulant activity of NETs in immunothrombosis.

INTRODUCTION

Protein Z-dependent protease inhibitor (ZPI) is a 72 kDa glycoprotein that is found at a concentration of 60 nM in the plasma. It belongs to the serpin superfamily and acts as a natural anticoagulant by specifically targeting the key procoagulants factor Xa (FXa) and factor XIa (FXIa). ZPI is so called because efficient inhibition of FXa requires the presence of protein Z (PZ), a vitamin K-dependent protein that (in the presence of phospholipid membrane surface and calcium) serves as a cofactor for ZPI[1–3]. ZPI also rapidly inhibits FXIa in a PZ-independent reaction, making ZPI the fastest known inhibitor of this factor in plasma[4]. In contrast to other serpins, ZPI forms an unstable complex with its target enzyme. Dissociation of the complex during the clotting process releases a proteolytically cleaved form of ZPI lacking a 4 kDa C-terminal peptide[1].

The physiological importance of ZPI in the regulation of coagulation is still subject to debate. ZPI knock-out mice have a mild thrombotic phenotype[5], whereas the conflicting results of clinical studies have failed to establish a robust association between ZPI deficiency and thrombophilia[6]. However, the results of the animal experiments and clinical studies suggest that alteration of the PZ/ZPI system exacerbates the prothrombotic state when it is combined with well-characterized thrombotic risk factors like the factor V Leiden mutation[5,7,8].

The anticoagulant ZPI might also be involved in other disease processes, such as cancer[9–14] and inflammation. In a murine model of inflammation, ZPI behaved as a typical acute phase reactant; the plasma ZPI antigen level and the liver ZPI mRNA level start rise a few days after the induction of local inflammation[15]. This elevation of the plasma ZPI antigen level under inflammatory conditions has also been observed

in clinical studies, where the plasma levels of ZPI, fibrinogen and C-reactive protein were significantly correlated [16,17].

Septic shock is the archetype clinical situation in which extensive crosstalk between inflammation and coagulation is observed[18]. The resulting abnormalities range from a slight activation of coagulation to overt disseminated intravascular coagulation (DIC)[19]. Sepsis-associated coagulopathies have been extensively documented. Although most coagulation factors and inhibitors are depleted during sepsis, the plasma levels of a few (such as fibrinogen, factor VIII, and von Willebrand factor) rise significantly during septic shock as markers of inflammation[20]. Furthermore, neutrophils are activated during sepsis; these cells release neutrophil extracellular traps (NETs) that form a procoagulant surface, promote fibrin formation, and thus ensnare invading pathogens[21]. The term “immunothrombosis” has been used to describe this cooperation between innate immunity and thrombosis[22]. However, the uncontrolled activation of immunothrombosis may be detrimental to the host and typically leads to septic shock-induced DIC[23]. The broad-spectrum protease neutrophil elastase (NE) is one of the principal components of the NETs’ procoagulant activity [24]. By catalyzing the proteolytic inactivation of tissue factor pathway inhibitor (TFPI, one of the main physiologic anticoagulants), NE thereby releases the brake on the procoagulant process[25]. Under normal conditions, coagulation is tightly regulated by physiological anticoagulants such as TFPI, antithrombin, and the protein C/protein S system. During sepsis, all these anticoagulant pathways are often impaired. Due to decreased protein synthesis, protein degradation, and coagulation factor depletion, the levels of natural anticoagulant are usually low - leading to an imbalance in the hemostatic system[26]. In a mouse model of lipopolysaccharide-induced systemic inflammation, ZPI-deficiency increases microvascular thrombi formation - suggesting

that an elevated level of ZPI is likely to be associated with a good clinical outcome in sepsis[27].

To the best of our knowledge, the impact of septic shock on the plasma ZPI antigen level has not previously been studied. In the context of severe sepsis, however, systemic inflammation and coagulation activation might have opposing effects on plasma ZPI antigen level. On one hand, and as an acute phase reactant, ZPI might be overexpressed. On the other hand, and as an anticoagulant, ZPI might be depleted during sepsis-induced coagulopathies. In this context, the objective of the present study was to measure changes in the plasma ZPI antigen concentration during septic shock.

MATERIALS AND METHODS

Materials

Polyclonal sheep anti-human ZPI antibodies (either conjugated to horseradish peroxidase (HRP) or not) were purchased from Molecular Innovation (Novi, MI, USA). NE purified from human leukocytes and human plasma α1-antitrypsin (α1-AT) were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Human PZ purified from plasma was purchased from HyphenBiomed (Neuville-sur-Oise, France). Human FXa and FXIa were from Stago BNL (Leiden, the Netherlands) and specific chromogenic substrates for FXa (CS-11(65)) and FXIa (PNAPEP-1566) were from HyphenBiomed and Cryopep (Montpellier, France), respectively. Phospholipid vesicles (phosphocholine:phosphoserine 3:1) were synthesized by membrane extrusion[28] in 10 mM HEPES (pH 7.5), 100 mM NaCl, 5 mM $CaCl_2$ buffer.

Production and purification of recombinant human ZPI (rhZPI)

Human ZPI cDNA (NCBI, NM_001100607.2) was cloned into the Not1 and BamH1 site of the pCEP4 vector (NeoBiotech, Nanterre, France). The plasmid pCEP4-ZPI was amplified in One Shot® TOP10 Chemically Competent *E. coli* (ThermoFisher Scientific, Illkirch, France) and transfected into FreeStyle 293-F cells (ThermoFisher Scientific) for rhZPI expression in cell culture media. Briefly, cells were grown in a humidified 8% CO_2 atmosphere at 37°C on an orbital shaker rotating at 125 rpm in F17 medium supplemented with 2 mM glutamine, 0.1 U/mL penicillin, and 0.1 mg/mL streptomycin. Cells (200 ml at 10^6 cells/mL) were transfected with 200 µg of pCEP4-ZPI combined with 400 µg of polyethylenimine (PEI 25K linear, Polysciences, Hirschberg an der Bergstrasse, Germany) in Opti-MEM® I medium (ThermoFisher Scientific). Seven

days after transfection, the cell culture media was harvested, frozen and stored (at -80°C) until rhZPI purification.

RhZPI was purified from 600 mL of conditioned media by heparin affinity chromatography on a 5-mL HiTrap Heparin HP column (Cytiva, Velizy-Villacoublay, France). After loading and washing with equilibrium buffer (20 mM MES buffer, 100 mM NaCl and 1 mM EDTA, pH 6.3), the RhZPI was eluted with a 20 mL linear gradient from 100 mM NaCl to 600 mM NaCl in the same buffer. Fractions containing rhZPI (as assessed by SDS-PAGE analysis) were pooled, dialyzed (dialysis buffer: 20 mM MES, 15 mM NaCl, 1 mM EDTA, pH 7.4) and loaded on a 1-mL RESOURCE Q column (Cytiva) equilibrated in the dialysis buffer. RhZPI was eluted with a 15 mL linear gradient from 15 mM to 150 mM NaCl in dialysis buffer and concentrated by ultrafiltration with a 30 KDa cut-off membrane in PBS. The rhZPI concentration was estimated by absorbance at 280 nM (absorption coefficient $\varepsilon = 1.44\ g^{-1}.L.cm^{-1}$), and the integrity of purified rhZPI was tested by SDS-PAGE. Lastly, rhZPI preparations were aliquoted and stored at -80°C before use in the functional assay.

Plasma samples from patients with severe sepsis and from healthy controls

Patients with septic shock (n=100, defined according to the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3)[29]) were prospectively enrolled (NCT 02391792). Two subgroups were defined according to the presence of absence of DIC, as diagnosed according to the Japanese Association for Acute Medicine score (JAAM-DIC 2006)[30] established within 48 hours of admission. Septic shock patients with a JAAM-DIC 2006 score below 4 were assigned to the non-DIC subgroup (n=62), while patients with the JAAM-DIC 2006 score of 4 or more were assigned to the overt DIC subgroup (n=38). Blood samples were collected in citrate tubes (9:1 v/v blood:3.2% sodium citrate) within 24 hours of their admission to the

intensive care unit. As a control, blood samples were also collected from healthy volunteers (n=31). Platelet-free plasma was prepared by double centrifugation (2500 g, 10 min), and the aliquots were stored at -80°C until they were analyzed.

PZ and ZPI antigen plasma levels

Plasma ZPI and PZ antigen levels were quantified in sandwich ELISAs. PZ antigen was assayed with a ZYMUTEST™ Protein Z ELISA kit (Hyphen BioMed), according to the manufacturer's instructions. ZPI antigen was measured using an in-house immunoassay, as follows: anti-ZPI capture antibody (5 µg/mL in 50 mM carbonate-bicarbonate coating buffer, pH 9.6) was coated in the wells of a 96-well, U-bottom microtiter plate (Nunc MaxiSorp™, ThermoFisher Scientific). After being washed with PBS containing 0.05% Tween-20 (PBST) and saturation with PBST containing 1% bovine serum albumin (BSA) for 1 hour at room temperature, samples diluted (1:800) in PBST were placed in the wells and incubated for 1 hour at room temperature. After washing with PBST, HRP-conjugated anti-ZPI detection antibody (5 µg/mL in PBST) was added to each well and incubated at room temperature for 1 hour. After washing, a solution of 3,3´,5,5´ tetramethylbenzidine (1-Step Turbo TMB-ELISA, ThermoFisher Scientific) was added to each well and incubated for 10 min before the addition of an equal volume of the stop solution (2 M sulfuric acid). Absorbance at 450 nm was then measured using a microplate reader (MP96, Safas, Monaco). The plasma ZPI antigen concentration was then determined according to a standard curve established with a pool of normal human plasma (Cryocheck™ Pooled Normal Plasma, Cryopep). The dose-response curve was linear at dilutions ranging from 1:100 to 1:2000. Each patient's plasma ZPI antigen concentration was expressed as a percentage of the plasma pool value.

Proteolysis of ZPI by purified NE

rhZPI (14 µM) was incubated with NE (4 or 40 mU/mL, as indicated) in 25 mM Tris pH 7.4 containing 137 mM NaCl, 2.7 mM KCl and 0.1% (w/v) polyethylene glycol-8000 (TBS-PEG) at 25°C. At the indicated incubation times, NE activity was quenched by the addition of 1:10 volume of phenylmethylsulfonylfluoride (10 mM, PMSF, Sigma-Aldrich). Samples were diluted in SDS-PAGE loading buffer for electrophoresis or in TBS-PEG containing 0.1% (w/v) BSA (TBS-PEG-BSA) and further incubated for 30 min at room temperature (the time required for degradation of PMSF and its inhibitory activity in aqueous buffer) for a chromogenic ZPI activity assay.

Neutrophil isolation and NETosis induction.

Neutrophils were isolated from fresh whole donor blood collected on acid-citrate-dextrose and provided by the Etablissement Français du Sang (Rungis, France), as described previously[31]. Briefly, leukocytes were separated from erythrocytes by sedimentation in 5% Dextran T-500 (SERVA Electrophoresis GmbH, Heidelberg, Germany) and 10% Radioselectan (Schering, Lys-lez-Lannoy, France) in 0.9% saline. Neutrophils were further separated from mononuclear cells by centrifugation on Pancoll Human (1.077 g/L, PAN-Biotech, Aidenbach, Germany). Contaminating erythrocytes were removed by hypotonic lysis. Neutrophils were resuspended in Hank's buffered salt solution (HBSS; Dominique Dutscher, Brumath, France) supplemented with 0.05% fetal calf serum (Dominique Dutscher) and 10 mM HEPES (PAN-Biotech). Neutrophil purity was assessed by observation of the nuclear morphology on a May-Grünwald-Giemsa-stained smear, and cell viability was assessed by Trypan Blue staining. Neutrophil purity and cell viability were always over 95% (data not shown).

Freshly isolated neutrophils were seeded in 48-well plates (5×10^5 cells/well, Dominique Dutscher) for analysis of rhZPI cleavage or an 8-chamber Lab-Tek® II Slide

system (2.5×10^5 cells/well, ThermoFisher Scientific) for immunostaining analysis. To induce NETosis, the cells were treated with 50 nM phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich) for 4 hours in a humidified 5% CO_2 atmosphere at 37°C. As a control of NETosis induction, the morphology of untreated neutrophils was compared with PMA-activated neutrophils after incubation (Supplementary Fig. S1).

Incubation of rhZPI with NETs

Four hours after the induction of NETosis with PMA, the cell culture medium was carefully removed and replaced with 100 µL of HBSS containing 1.4 µM rhZPI alone or in the presence of 10 µM α1-AT or 1.6 µM PZ. After incubation in a humidified 5% CO_2 atmosphere at 37°C for different periods of time (from 15 to 90 minutes), ZPI-containing supernatants were collected and immediately spiked with PMSF (1 mM final). Next, NETs were either washed with PBS and fixed with 4% (w/v) paraformaldehyde (PFA) solution in PBS for 30 min (for immunostaining) or treated with 20 U/L DNAse-I (Promega) in HBSS supplemented with 10% v/v 10X DNase reaction buffer and 1 mM PMSF (100 µL/well) for 10 min at 37°C. Samples containing dismantled NETs were also collected and centrifuged (16000g, 10 min) to remove cell debris. Lastly, ZPI-containing samples were either prepared for SDS-PAGE by dilution in SDS-PAGE loading buffer or frozen for chromogenic ZPI activity assays.

Time course of protease inhibition by rhZPI

The time course of protease inhibition by rhZPI (after treatment with NE or incubation with NETs) was measured in TBS-PEG-BSA in continuous chromogenic assays under pseudo-first-order conditions at 25°C. FXa inhibition was initiated by the addition of human FXa (400 pM) to a mixture containing rhZPI (70 nM), PZ (100 nM), phospholipid vesicles (25 µM), the FXa specific chromogenic substrate CS-11(65) (300 µM), and

$CaCl_2$ (5 mM). FXIa inhibition was initiated by the addition of human FXIa (400 pM) to a mixture containing rhZPI (500 nM) and the FXIa-specific chromogenic substrate PNAPEP-1566 (200 µM). Hydrolysis of the substrate was measured at 405 nm using a microplate reader (MP96, Safas). When that was possible, the apparent rate constant for proteases inhibition by ZPI was estimated by fitting the progress curves of substrate hydrolysis to the equation for slow-binding inhibition[32].

SDS-PAGE and Western blots

ZPI-containing samples were prepared in Laemmli SDS-PAGE loading buffer in the absence of reducing agent and then subjected to electrophoresis on Bis-Tris Novex™ NuPAGE™ 4-12% gels in NuPAGE™ MOPS-SDS Running Buffer (ThermoFisher Scientific). Protein bands were either stained with InstantBlue™ (VWR, Fontenay-sous-Bois, France) according to the manufacturer's instructions, or transferred to a nitrocellulose membrane and immunoblotted with a polyclonal sheep anti-human ZPI antibody (0.5 µg/mL, Molecular Innovation) and revealed by HRP-conjugated anti-sheep secondary antibody (0.05 µg/ml, Jackson ImmunoResearch Europe Ltd, Ely, UK) and chemiluminescence. Band intensity was quantified using ImageJ software (NIH, Bethesda, MA, USA).

Plasma samples were diluted 50-fold in SDS-PAGE loading buffer and loaded (15 µL/lane) for Western blotting. For samples containing rhZPI incubated with purified NE or NETs, the total amount of rhZPI loaded was 3 µg per lane for InstantBlue™ staining and 25 ng per lane for Western blotting.

Immunostaining

After fixation with PFA, NETs were washed (three times) with PBS, blocked with 1% BSA in PBS over night at 4°C, and incubated first with 20 µg/mL anti-human ZPI

antibody (Molecular Innovation) in PBS for 30 min at room temperature and then with 2 µg/mL Alexa Fluor 594 donkey anti-sheep IgG (ThermoFisher Scientific) in PBS for 30 min at room temperature. The slides were mounted in medium containing 4’,6-diamidino-2-phenyl indole (DAPI, SouthernBiotech, Birmingham, AL, USA) to label DNA, and widefield microscopy images were acquired on an AxioImager A1 system (Carl Zeiss, OberKocher, Germany).

RESULTS

Analysis of plasma ZPI antigen in a cohort of patients with septic shock

Plasma ZPI antigen levels were measured (using an immunoassay) in a cohort of patients with severe sepsis on admission to the intensive care unit and (as a control) in a group of healthy blood donors (Table 1). As expected, the mean ± standard deviation (SD) ZPI antigen levels were approximately 2.5-fold higher in the septic shock group (9.91 ± 4.83 µg/mL) than in the control group (3.89 ± 1.41 µg/mL; $p < 0.0001$). In contrast, the mean plasma PZ level was significantly lower in the septic shock group (1.27 ± 0.96 µg/mL) than in control group (2.07 ± 0.88 µg/mL; $p < 0.0001$). Hence, the ZPI:PZ molar ratio was ~1.6 in healthy donors and ~6.7 in patients with septic shock. The sepsis group was further divided in two subgroups, according to the presence or absence of DIC. Interestingly, the difference in plasma ZPI antigen concentration between the non-DIC and DIC subgroups (9.61 ± 3.48 and 10.40 ± 6.49 µg/mL) was not statistically significant, even though both values were significantly greater ($p < 0.0001$) than that of the control group. The same was true for PZ, with similarly low plasma levels in the non-DIC and DIC subgroups (1.34 ± 1.01 µg/mL and 1.16 ± 0.88 µg/mL, respectively). However, the plasma level of ZPI antigen does not necessary reflect ZPI activity. It is known that ZPI is depleted when it is involved in coagulation, with the release of a cleaved form of ZPI lacking its 4-kDa C-terminal peptide[1]. Hence, some plasma samples from the sepsis group were also analyzed by Western blotting and compared with normal human plasma and serum (Figure 1 and Supplementary Fig. S2). ZPI in plasma from healthy donors appeared as a single band at the expected molecular weight of the native protein (~72 kDa), while ZPI in plasma from septic shock patients migrated as a two bands whose apparent molecular weights corresponded to native ZPI and cleaved ZPI, respectively, as observed in control

serum. Thus, the lower band in plasma from septic shock patients corresponded to the cleaved form of ZPI, probably lacking its ~4 kDa C-terminus peptide after a cleavage within its reactive center loop (RCL). It is noteworthy that the two bands were observed in all the plasma samples from the septic shock group, independently of the DIC status. Quantification of the band intensity revealed that the proportion of cleaved ZPI was similar in the non-DIC and DIC subgroups (12.9 ± 4.3%, n = 12 and 11.9 ± 4.7%, n=13, respectively). Hence, the presence of cleaved ZPI was probably not related to the activation of coagulation - thus raising the question of the origin of cleaved ZPI in plasma samples from severe sepsis patients. Given that NE is released from neutrophils during inflammation (either due to neutrophil degranulation or to NETosis) and NE is known to catalyze proteolytic inactivation of the main natural anticoagulants (such as TFPI[25], antithrombin[33], and protein S[34]), we hypothesized that NE is part of the process leading to the cleavage of ZPI.

ZPI inactivation by neutrophil elastase

rhZPI was incubated with purified NE, and the time course of proteolytic cleavage was examined using SDS-PAGE (Figure 2A and B). The results indicated that intact rhZPI was initially cleaved to generate a cleaved form of rhZPI with an apparent molecular weight 4 kDa below that of intact rhZPI. This initial cleavage occurred rapidly - in less than 2 min when rhZPI was incubated with a high NE concentration (40 mU/mL) or in less than 40 min in the presence of a low NE concentration (4 mU/mL). After prolonged incubation of rhZPI with 40 mU/mL NE, two additional cleavage products were observed, with apparent molecular weights of ~58 and ~47 kDa, respectively. N-terminal sequencing of the three cleaved forms of rhZPI revealed that the initial cleavage occurred in the C-terminal region of rhZPI, since the N-terminal sequence of the 68 kDa cleavage product matched that of native rhZPI (LAPSPQ). The two

subsequent cleavages occurred in the N-terminal region of rhZPI (after the residues Val 20 and Ile 103, respectively).

The inhibitory activity of rhZPI after NE-catalyzed proteolysis was also assessed by measuring (i) its anti-FXa activity in the presence of PZ, phospholipids and calcium (Figure 2C-D), and (ii) its anti-FXIa activity in the absence of cofactor (Figure 2E-F). We found that within the first few minutes of the reaction with 40 mU/mL NE concentration, rhZPI no longer inhibited the amidolytic activities of FXa or FXIa. The loss of inhibitory activity was slower when rhZPI was incubated with a low NE concentration. For both concentrations of NE, the time course of the loss of rhZPI activity (Figure 2G) coincided with that of the 4-kDa peptide cleavage observed by SDS-PAGE. This finding implies that the NE-catalyzed proteolysis of ZPI in its C-terminal region induces a concomitant loss of anticoagulant activity.

Because other proteases are stored in neutrophil granules and released upon neutrophil activation, we have also investigated the impact of cathepsin G and proteinase 3 on proteolytic inactivation of ZPI in vitro (Supplementary Fig. S3). Although a subtle shift of its electrophoretic feature was evidenced, ZPI tended to lose its anticoagulant activity upon incubation (10 min) with increasing concentrations of cathepsin G, while it remained resistant to proteinase 3-catalyzed proteolysis. Nevertheless, cathepsin G concentration required to achieve a complete ZPI inactivation was higher than that of NE, suggesting that among neutrophil enzymes, NE is the principal inhibitor of ZPI.

ZPI inactivation by NETs

In view of the impact of immunothrombosis during sepsis, we evaluated the NETs' effect on rhZPI activity. To this end, NETs were produced in vitro by PMA stimulation

of isolated human neutrophils for four hours. After NETosis, rhZPI was added to the cell culture medium. In a first series of experiments, the binding of rhZPI to NETs was analyzed by immunofluorescence (Figure 3). Immunostaining analysis revealed the colocalization of rhZPI with DAPI-stained extracellular DNA fibers, and thus indicated that rhZPI can bind to NETs. A similar binding pattern was observed even when the complex PZ/rhZPI was pre-formed prior to incubation with NETs. In a second set of experiments, rhZPI was incubated with NETs in the presence or absence of the specific NE inhibitor α1-AT. We analyzed the conformational and functional status of rhZPI in the supernatants collected after various incubation times. In the absence of α1-AT, native rhZPI (the ~72-kDa band) was rapidly converted into a smaller, cleaved form with an apparent molecular weight of ~68 kDa. This cleavage occurred within the first 15 minutes of incubation with NETs (Figure 4A) and no additional cleavage was observed over the duration of the experiment (up to 90 min). Furthermore, the incubation of rhZPI with NETs resulted in the loss of its anticoagulant activity, as evidenced by the inability of the rhZPI in the NET supernatant to inactivate the amidolytic activities of FXIa (Figure 4B) and FXa (Supplementary Fig. S4). In contrast, when rhZPI was incubated with NETs in the presence of α1-AT, the electrophoretic profile of rhZPI did not change over the course of the experiment (Figure 4C), and the rhZPI retained its ability to inhibit FXIa (Figure 4D) and FXa (Supplementary Fig. S4). Indeed, the rate of FXIa inhibition by ZPI incubated with NETs in the presence of α1-AT up to 60 minutes remained similar to that measured when FXIa was subject to inhibition by ZPI alone, prior incubation with NETs (mean ratio of apparent rate constants = 1.01 ± 0.10). Nonetheless, when ZPI was combined with α1-AT prior incubation with NETs, the rate of FXIa inhibition appeared slightly increased (mean ratio of apparent rate constants = 1.73 ± 0.47). This might be explained by the high α1-

AT concentration used in this experiment (10 µM) that could contribute to FXIa inhibition. This interference of α1-AT was subsequently reduced once the mixture was incubated with NETs as a result of its engagement in a covalent complex with NE. Taken as a whole, these results suggest that the proteolytic inactivation of rhZPI observed after incubation with NETs is mainly due to NE activity released upon NETosis. Indeed, the effect of NETs on rhZPI was very similar to that observed when rhZPI was incubated with purified NE alone, and was inhibited by α1-AT.

After incubation of rhZPI with NETs and removal of the rhZPI-containing supernatant, NET-bound proteins were collected after treatment with DNase. Western blotting of the proteins extracted from the dismantled NETs revealed the presence of rhZPI, which remained bound to the NETs. In the absence of α1-AT, NET-bound rhZPI underwent at least two cleavages: the first led to the cleaved form of rhZPI (~68 kDa), and the second led to the truncated form of rhZPI (~58 kDa) – the same as described above following the reaction between purified rhZPI and a high NE concentration. Remarkably, NET-bound rhZPI appeared to be cleaved even in the presence of α1-AT, and the migration pattern corresponded to that of cleaved rhZPI (~68 kDa). This might be explained by the combination of a greater concentration of NE activity in the vicinity of the NETs and incomplete inhibition of NET-bound NE by α1-AT (Figure 4E and F).

DISCUSSION

During septic shock, abnormal coagulation and systemic inflammation might be associated with a poor clinical outcome. Whether as a cause or a consequence of these perturbations, levels of natural anticoagulant are usually low in patients with severe sepsis[26]. According to the literature, ZPI is an acute-phase reactant whose plasma concentration increases under inflammatory conditions[15–17]. Overexpression of anticoagulant ZPI can be viewed as a beneficial response that counters the procoagulant process during sepsis. This thought is what initially prompted us to evaluate the plasma level of ZPI in a cohort of patients suffering from severe sepsis. Our result confirmed that the plasma ZPI antigen concentration is elevated during sepsis and that ZPI behaves as an acute-phase reactant. However, we also found that plasma ZPI in patients with sepsis appears to be partially truncated. Unlike plasma ZPI from healthy donors (which migrated as a single band on SDS-PAGE), ZPI from "sepsis plasma" migrated as two bands: the upper band corresponded to native ZPI and the lower band corresponded to the cleaved form of ZPI (also observed in serum). Serpins usually lose their inhibitory activity upon cleavage, with their RCL buried in β-sheet A[35]. We therefore suspected that the cleaved ZPI found in sepsis plasma had lost its anticoagulant activity. Unfortunately, the lack of a reliable assay of ZPI anticoagulant activity in plasma prevented us from measuring any residual activity. Hence, we were unable to determine whether ZPI's anticoagulant activity rises in parallel with the elevated antigen level or decreases due to partial cleavage of ZPI in plasma from patients with severe sepsis.

Furthermore, the evaluation of ZPI's anticoagulant activity during sepsis would be of great interest with regard to its dual inhibitory effect on FXa (requiring the presence of PZ) and FXIa (whose enzymatic activity is mainly modulated by free ZPI[1]). Our results

showed that the rise in ZPI coincides with a drop in PZ antigen levels, causing a ~10-fold increase in the free ZPI:PZ-bound ZPI ratio in plasma from patients with severe sepsis (assuming that PZ bound fully to ZPI). In turn, this would impair ZPI's anti-FXa activity but might also enhance its anti-FXIa activity. FXI is a key component of the intrinsic coagulation pathway that is probably involved in immunothrombosis and in sepsis-induced coagulopathies. The literature data demonstrate that FXI gene deletion in mice does not exacerbate a bleeding tendency but increases survival during sepsis by reducing sepsis-associated coagulopathy[36,37]. Hence, an inflammatory-mediated rise in ZPI that would tun-off FXIa activity would be an asset in countering sepsis.

During the clotting process, ZPI reacts with FXa and FXIa. Due to the instability of the serpin-enzyme complex, however, proteolytic cleavage of ZPI's RCL releases a 4-kDa C-terminal peptide[1]. Since severe sepsis is frequently associated with abnormal coagulation, it was initially thought that the cleaved ZPI in the plasma of patients with sepsis resulted from ZPI consumption during sepsis-induced coagulation. Unexpectedly, however, the level of cleaved ZPI observed in the plasma of sepsis patients was not correlated with the intensity of coagulation activation, which ranged from a slight activation of coagulation to fulminant DIC. Indeed, our non-DIC and DIC subgroups (defined by JAAM-DIC 2006 scores of <4 and ≥ 4, respectively) had similar proportion of cleaved ZPI (~10% of the total ZPI).

We therefore hypothesized that the cleaved ZPI in the plasma of septic shock patients had been generated by a proinflammatory mechanism rather than by the activation of coagulation. We also wondered whether (as seen for other serpins, such as antithrombin or plasminogen activator inhibitor-1) ZPI was a substrate for inflammatory enzymes like NE[33,38]. In line with our hypothesis, purified ZPI indeed underwent several proteolytic cleavages when incubated with purified NE. The first cleavage led to a form

of ZPI that had lost a 4-kDa peptide from its C-terminal region and thereby also lost its anticoagulant activity. This observation suggested that NE catalyzes ZPI proteolysis within its RCL, leading to the thermodynamically stable form of the serpin in which the RCL is inserted into the β-sheet A. Peptide sequence analysis predicts four potential nicking sites for NE within ZPI's RCL (Ala376, Val377, Ala378, and Ala386); these are very close to the cleavage sites for FXa and FXIa (Tyr387). The low-molecular-weight peptides resulting from ZPI's reaction with FXIa or NE have similar apparent masses on SDS-PAGE (data not shown) but are not recognized by the polyclonal antibody against ZPI, and thus escape detection for further mass spectrometry analysis. As a consequence, the two cleaved forms of ZPI (originating from either depletion during coagulation or inactivation by NE) differ by a few amino acids only and are therefore undistinguishable - especially in plasma.

ZPI's sensitivity to NE-catalyzed inactivation might be clinically relevant in cases of sepsis and immunothrombosis. It is now well established that NETs are released during systemic inflammation and might retain their protease activity in this context. Although a high plasma concentration of α1-AT would inhibit circulating NE very quickly (a few ms), DNA-bound NE appears to resist α1-AT inhibition *in vitro*[39,40]. Our present results showed that ZPI can bind to NETs and that NE-catalyzed proteolytic inactivation of ZPI also occurs on the surface of NETs *in vitro*. Given that free ZPI is protected from NE-catalyzed proteolysis after incubation with NETs in the presence of α1-AT but NET-bound ZPI is rapidly cleaved (even in the presence of a high α1-AT concentration), we hypothesized that the NET surface favors the inactivation of ZPI - probably by hindering local inhibition of NE activity. A recent *in vivo* study found that NET-associated NE remains active within the vasculature[41] and might therefore be involved in the proteolytic inactivation of ZPI observed in plasma from patients with

severe sepsis. This finding provides a novel mechanism for the procoagulant activity of NETs. Furthermore, NETs may also disrupt anticoagulant pathways involving antithrombin, thrombomodulin or protein S, which are also known to be cleaved and inactivated by NE. Taken as a whole, our results suggest that the NE present in NETs has a broad spectrum of action and impairs most of the natural anticoagulant. However, the origin of the cleaved ZPI observed in the plasma of patients with septic shock remains uncertain and requires further investigation.

DISCLOSURES

The authors declare that they have no competing financial interests.

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FIGURE LEGENDS

Figure 1

Western blot analysis of plasma ZPI from septic shock patients:

Electrophoretic features of ZPI in plasma from septic shock patients with overt DIC (lanes 1 to 5) or without DIC (lane 6 to 10) or a healthy donor (lane 11), and in kaolin-treated plasma from the same healthy donor (lane 12). The bands corresponding to native or cleaved ZPI are indicated by arrows.

Figure 2

***In vitro* cleavage of rhZPI by NE:**

Purified rhZPI (14 µM) was incubated with purified NE (40 mU/mL (A, C, and E) and 4 mU/mL (B, D, and F)). At the time points indicated on the figures, a sample was withdrawn from the reaction mixture and spiked with PMSF (final concentration: 1 mM). In a first set of experiment, the samples (rhZPI: 3 µg/lane) were analyzed using SDS-PAGE (A and B). The dashed line indicates the migration feature of native rhZPI, with an apparent molecular weight of ~72 KDa. In a second set of experiments, the samples were incubated at room temperature until free PMSF had been inactivated. rhZPI's residual -anti-FXa (C and D) or -anti-FXIa (E and F) activity was then measured in a continuous chromogenic assay, as described in the Materials and Methods section. The graphs (C, D, E, and F) represent the time course of chromogenic substrate hydrolysis by proteases, in the absence of rhZPI (solid line) or in the presence of rhZPI incubated with NE for 0 (●), 2 (○), 5 (■), 10 (□), 20 (▲), 40 (△), or 60 (×) minutes. The panel G shows the residual ZPI anti-FXa (●, ○) and anti-FXIa (■, □) activities as the function of incubation time with 40 mU/mL (●, ■) and 4 mU/mL (○, □) NE. Each

data point corresponds to the mean (± SD) of three distinct experiments conducted in the same conditions.

Figure 3

Binding of rhZPI to NETs, as revealed by immunofluorescence staining:

To induce NETosis, human neutrophils were seeded in an 8-well chamber Lab-Tek® II Slide system at a density of 2.5 × 10^5 cell/cm^2 and stimulated with 50 nM PMA for 4 hours. After NET formation, rhZPI (1.4 µM) and PZ (1.6 µM), as indicated on the figure, were added to the cell culture medium and further incubated for 30 min. The cell culture medium was then removed, and the NETs were washed once with PBS and fixed with PFA. ZPI was revealed by immunofluorescence staining with a specific polyclonal sheep primary antibody against human ZPI and Alexa 594 anti-sheep secondary antibody. DNA was stained with DAPI mounting medium. This figure is representative of at least four experiments carried out with neutrophils from different healthy donors.

Figure 4

Proteolytic inactivation of rhZPI incubated with NETs:

100 µL/well of rhZPI (1.4 µM) were incubated with NETs in the absence of α1-AT (Figure 4A, B, and E) or presence of α1-AT (10 µM; Figure 4C, D, and F). At the indicated time points, supernatants containing soluble rhZPI were collected and the NETs were immediately treated with 100 µL/well of DNase (20 U/mL). After a 10 min incubation (to dismantle NETs), the supernatant containing NET-bound rhZPI was collected. Samples containing soluble rhZPI were diluted 1/20 in 1X SDS-PAGE loading buffer (Figure 4A and C), while samples containing NET-bound rhZPI were diluted 1/2 in 2X SDS-PAGE loading buffer (Figure 4E and F). 5 µL of each sample were subjected to electrophoresis for Western blotting using a sheep polyclonal

antibody against human ZPI. The residual anti-FXIa activity of ZPI was also measured in the supernatants containing soluble rhZPI, by using a continuous chromogenic assay as described in the Materials and Methods section (Figure 4B and D). The graphs represent the time course of chromogenic substrate hydrolysis by FXIa in the absence of rhZPI (solid line) or in the presence of rhZPI before incubation with NETs (●) or after 15 (○), 30 (■), or 60 (□) minutes of incubation with NETs. This figure is representative of at least three experiments carried out with neutrophils from different healthy donors.

TABLE

Table 1: Plasma ZPI and PZ antigen levels in study participants

	Sepsis (all patients)	Sepsis with overt DIC	Sepsis with non-overt DIC	Controls
N	100	38	62	31
ZPI µg/mL (mean, SD)	9.91 ± 4.83**	10.40 ± 6.49**	9.61 ± 3.48**	3.89 ± 1.41
ZPI % (mean, SD)	247.80 ± 120.80**	260.00 ± 162.20**	240.40 ± 87.10**	97.12 ± 35.22
PZ µg/mL (mean, SD)	1.27 ± 0.96**	1.16 ± 0.88**	1.34 ± 1.01*	2.07 ± 0.88
PZ % (mean, SD)	56.57 ± 42.77**	51.57 ± 38.89**	59.63 ± 45.02*	92.11 ± 38.95
ZPI/PZ (molar ratio)	6.7	7.7	6.2	1.6

Table 1: Plasma ZPI and PZ antigen levels in study participants:

Plasma ZPI and PZ antigen levels were quantified using immunoassays, as described in the Materials and Methods section. Relative concentrations were determined according to a standard curve established using a pool of normal human plasma and expressed as a percentage of the latter. Absolute concentrations were then calculated by assuming that the ZPI and PZ antigen concentrations in the pooled plasma were 4 µg/mL and 2.25 µg/mL, respectively. Data are quoted as the mean ± standard deviation. Statistical analysis was performed using a Kruskal-Wallis test (*and ** indicate that the mean is significantly different from the corresponding mean in the control group, with $p < 0.0002$ or 0.0001, respectively). The ZPI:PZ molar ratio was calculated using molecular weights of 72 KDa and 62 KDa, respectively.

FIGURES

Figure 1

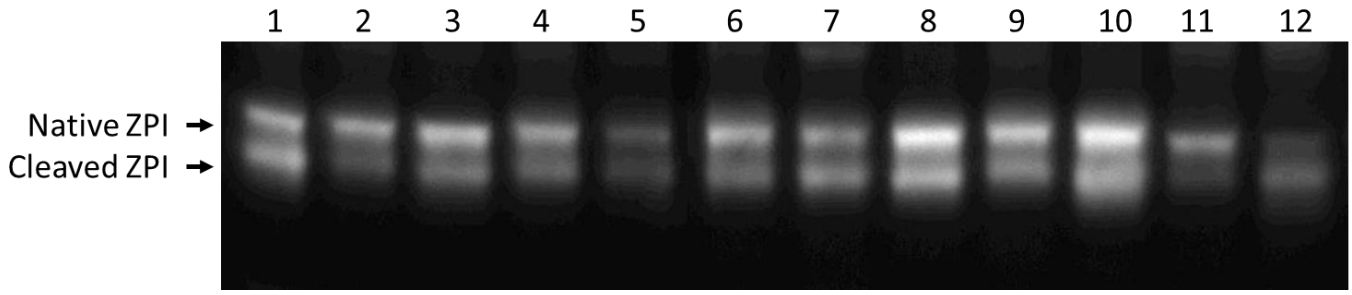


Figure 2

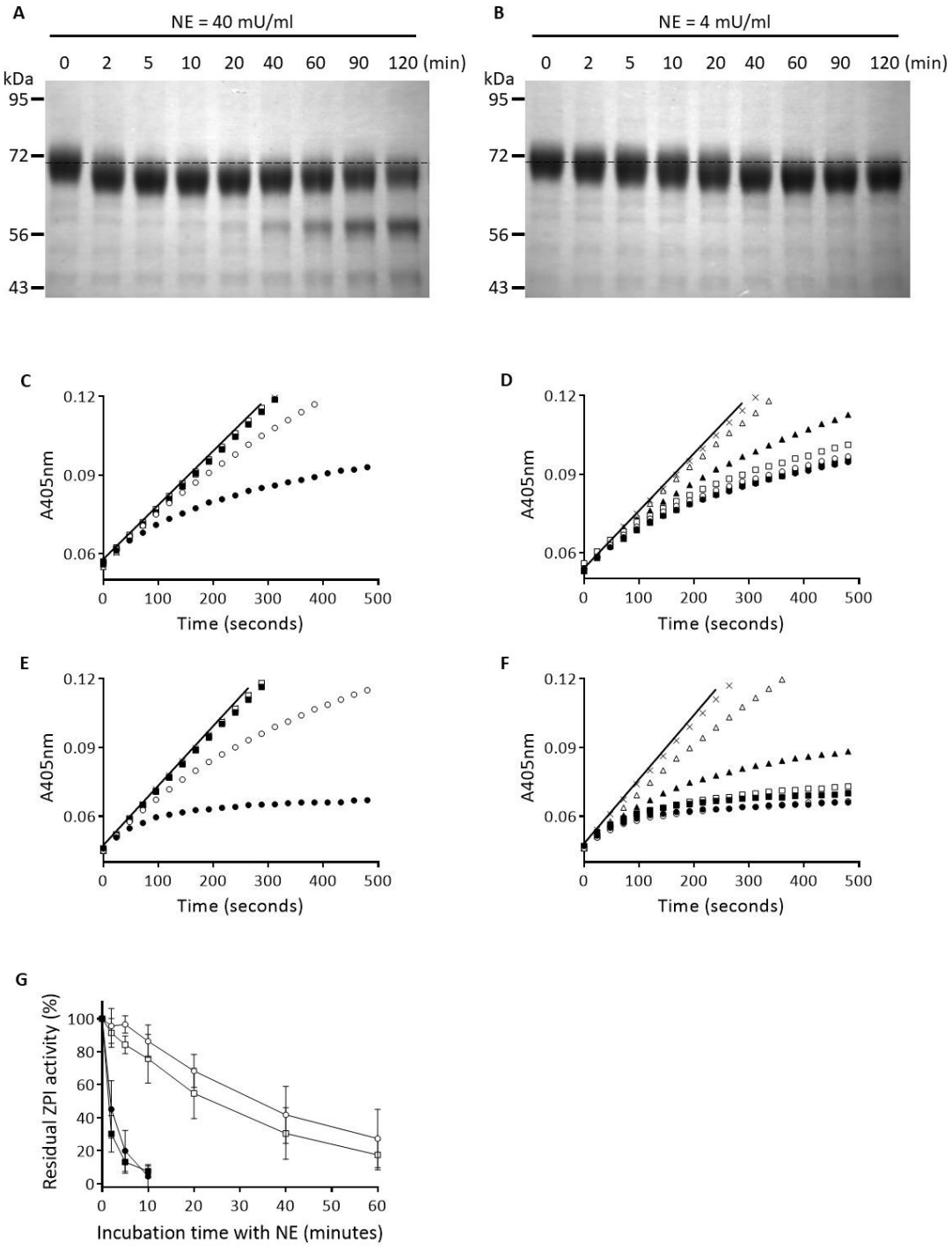

A
NE = 40 mU/ml
0 2 5 10 20 40 60 90 120 (min)
kDa
95
72
56
43
B
NE = 4 mU/ml
0 2 5 10 20 40 60 90 120 (min)
kDa
95
72
56
43
C
A405nm
0.12
0.09
0.06
0 100 200 300 400 500
Time (seconds)
D
A405nm
0.12
0.09
0.06
0 100 200 300 400 500
Time (seconds)
E
A405nm
0.12
0.09
0.06
0 100 200 300 400 500
Time (seconds)
F
A405nm
0.12
0.09
0.06
0 100 200 300 400 500
Time (seconds)
G
Residual ZPI activity (%)
100
80
60
40
20
0
0 10 20 30 40 50 60
Incubation time with NE (minutes)

Figure 3

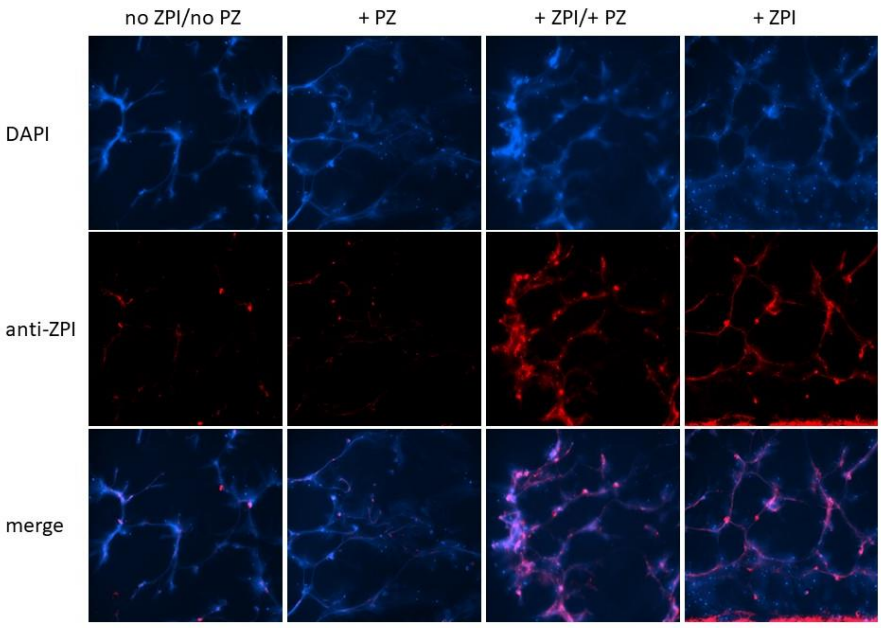
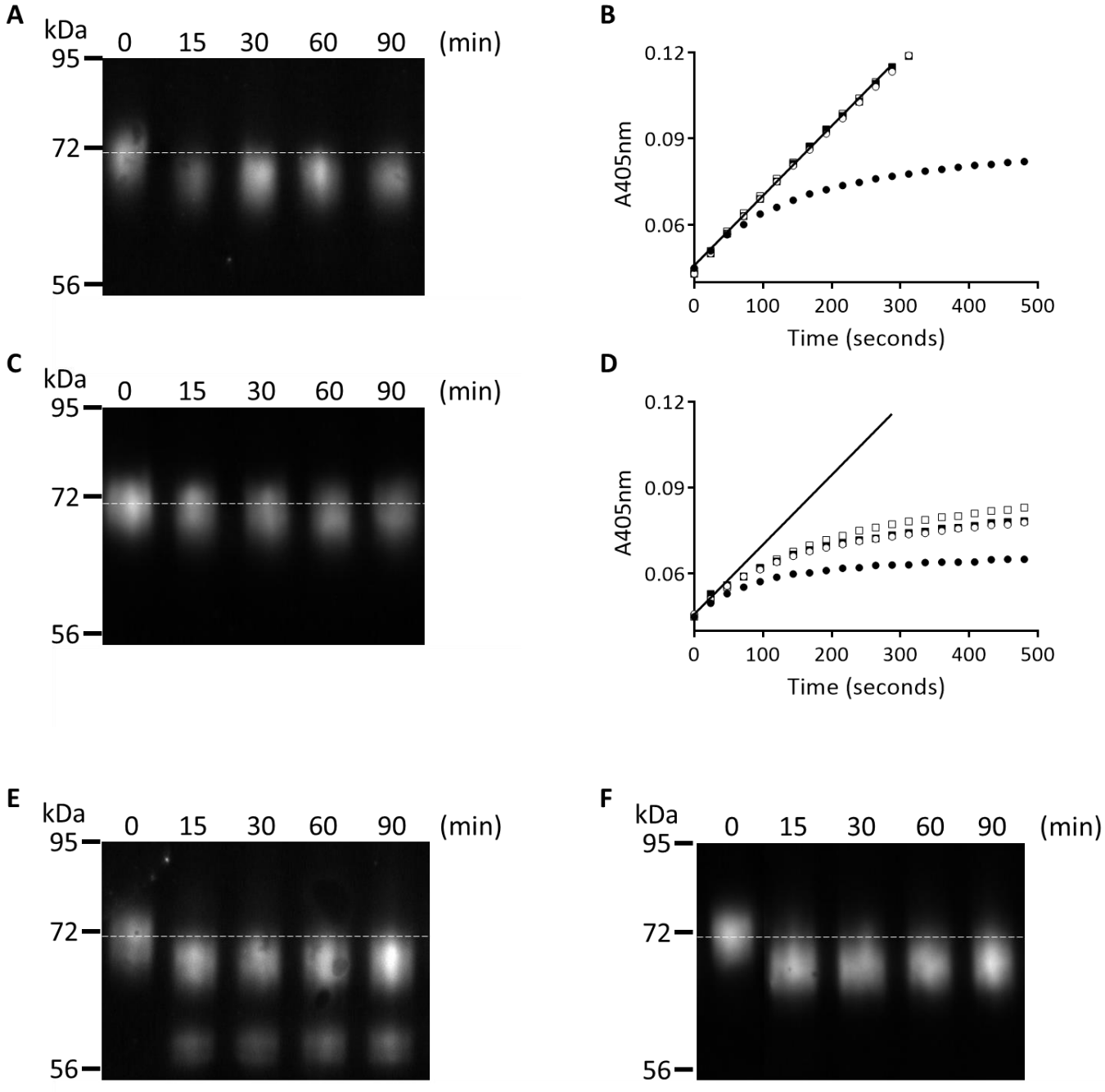


Figure 4


A
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0 15 30 60 90 (min)
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D
A405nm
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0 100 200 300 400 500
Time (seconds)
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0 15 30 60 90 (min)
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72
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F
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