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Potentiation of TLR4 signalling by plasmin activity

Jon R. Ward, Steven K. Dower, Moira K.B. Whyte, David J. Buttle, Ian Sabroe *

Division of Genomic Medicine, University of Sheffield Medical School, University of Sheffield, Sheffield S10 2JF, UK

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Abstract

The potential for proteases to regulate mammalian TLR signalling is controversial. We found that inhibition of extracellular serine proteases did not reduce activation of TLR4, but observed that the protease plasmin, an important fibrinolytic plasma enzyme that also exerts proinflammatory functions in monocytes, potentiated TLR2 and TLR4 signalling in RAW264.7 macrophages. Plasmin enhanced endogenous production of TNFα and activation of an NF-κB reporter plasmid. These actions were prevented by inhibition of its proteolytic activity and were not recapitulated by agonists of protease-activated receptors. These studies link fibrinolysis and TLR signalling, identifying further mechanisms potentially involved in activation of innate immunity.

Keywords: Toll-like receptor; Plasmin; LPS; Macrophage; Priming; PAR

Toll-like receptors are crucial to mediation of a response to bacterial-derived molecules. Constituents of Gram-negative bacterial cell walls activate TLR4 (LPS) and TLR2 (lipoproteins), whilst Gram-positive cell wall constituents predominantly activate TLR2 (lipoproteins, lipoteichoic acids) [1]. The *Drosophila* homologue, Toll, is also activated by pathogens, but via an intermediate step, dependent upon proteases that cleave a pro-ligand to generate an active Toll ligand, Spaetzle [2,3]. The role of extracellular proteases in TLR signalling is less clear. One study reported that responses to LPS in the human monocyte cell line THP-1 were inhibited by the serpin (serine protease inhibitor) antithrombin III (ATIII), acting on a non-thrombin serine protease potentially analogous to the signalling system of *Drosophila* [4]. In contrast, other work demonstrated that protease inhibitors prevented LPS signalling in epithelial and endothelial cells, but only as a result of inhibiting cleavage of IκB, a prerequisite for the mobilisation of NF-κB [5,6]. C1 inhibitor (another serpin) also inhibits LPS signalling, but by binding to LPS and preventing its recognition by TLR4 [7]. Given the need to have effective innate immunity and effective clotting at wound sites, close links between the clotting cascade and innate immunity are unsurprising. Fibrinogen can activate TLR4 [8], and urokinase-type plasminogen activator (UPA) can potentiate LPS responses in neutrophils [9]. The potent fibrinolytic protease plasmin causes generation of cytokine production by monocytes [10,11]. In this study, we investigated the potential for proteases to influence TLR responses in monocytic cells and focused on the ability of plasmin to modulate LPS signalling.

Materials and methods

Materials. LPS from *Escherichia coli* strain 0111:B4 was from Sigma–Aldrich (Poole, UK). Pam_3CSK_4 (an agonist of TLR2) was from EMC Microcollections (Tübingen, Germany). Plasmin (EC 3.4.21.7) was from two suppliers: Merck Biosciences (Nottingham, UK) and Fluka (Poole, UK). Thrombin (EC 3.4.21.5) was from Merck Biosciences. Endotoxin contamination of plasmin preparations was measured commercially (Cambrex Bioscience). The protease inhibitors leupeptin and D-Val-Phe-Lys-chloromethyl ketone (VPLK) were from Sigma and Merck Biosciences. Antithrombin III (ATIII) and urokinase-type plasminogen activator (uPA) were from MP Biomedicals (Irvine, CA) and Chemicon International (Chandlers Ford, UK). Agonists of PAR-1 (sequence SFLLRN, human- and mouse-activating) and PAR-2 (sequence SLIGRL, mouse-specific) were from Bachem (St. Helens, UK).

Verification of protease activity. Proteases and inhibitors were tested for their ability to cleave or inhibit cleavage of fluorescent substrates (Bachem) in assay buffer (50 mM Tris, 5 mM $CaCl_2$, and 0.01% Chaps, pH

* Corresponding author. Fax: +44 114 272 1104.
E-mail address: i.sabroe@sheffield.ac.uk (I. Sabroe).

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7.5), measured at 37 °C in a continuous assay using a Perkin-Elmer LS-50B spectrophotometer with FLU-SYS software [12]. Substrates were: Boc-Val-Pro-Arg-aminomethylcoumarylamide (AMC)·HCl (for thrombin), Z-Gly-Gly-Arg-AMC·HCl (for uPA), and Suc-Ala-Phe-Lys-AMC.TFA (for plasmin), and were used at 5 μM. Final calculation of units/ml for dilutions of proteases was based on the manufacturer's supplied data.

Cell lines and functional assays. The RAW264.7 mouse macrophage cell line was maintained in Dulbecco's modified Eagle's medium with 10% FCS. Cells were seeded in 96-well plates (50,000 cells/well) 24–48 h before transfection (using FUGENE 6 (Roche, Basle, Switzerland)) with the pIL-8 luc NF-κB reporter plasmid [13] (100 ng/well) and the pTK-Rluc *Renilla* luciferase plasmid (100 ng/well, Promega, Southampton, UK) providing a control for transfection efficiency. Twenty-four hours later, cells were treated with agonists as indicated. Five hours after the addition of LPS, generation of luciferase was determined using the dual luciferase reagent system (Promega). In the indicated experiments, plasmin was added one hour prior to addition of LPS (without removal of plasmin) for a further five hours. Where protease inhibitors were used, a 10× stock concentration of plasmin was pretreated with leupeptin (100 μM) or VPLK (50 μM) for 30 min, after which the enzyme/inhibitor mix was added to the cells (1:10 dilution). NF-κB-driven luciferase levels were normalised to the pTK-Rluc data to control for transfection efficiency.

In similar experiments, untransfected RAW cells were stimulated with plasmin and/or the indicated agonists and inhibitors, cell-free supernatants prepared, and generation of TNFα determined using a murine TNFα ELISA (R&D Systems, Abingdon, UK), with a lower limit of detection taken as 125 pg/ml (this value being used where cytokine levels in samples were undetectable).

Data were analysed using the GraphPad Prism v4.0b program (GraphPad.com, San Diego, CA).

Results

The role of serum in LPS responses

Serum contains many potent proteases and protease inhibitors, yet also contains LPS binding protein (LBP) and soluble CD14, which are thought to play important roles in the presentation of LPS to cell surface TLR4. In order to allow an investigation of the roles of proteases in LPS responses, we investigated the requirement of serum for LPS responses in RAW macrophages. LPS induced activation of the NF-κB reporter in the presence and absence of serum (Fig. 1A), though the concentration–response curve showed a rightward shift in the absence of serum. In contrast, responses to Pam_3CSK_4 were not influenced by removal of serum (Fig. 1B). Concentration–response relationships of LPS activation of RAW cell batches were monitored throughout this work. The ability of proteases and inhibitors to modify LPS responses was tested at submaximal LPS concentrations, in the absence of serum, these values being typically 10 or 30 ng/ml LPS.

Direct activation of monocytes by plasmin

We investigated the potential of the proinflammatory plasma protease, plasmin, to activate RAW cells. A five-hour treatment with plasmin caused a modest activation of the NF-κB reporter (Fig. 2), in keeping with the known ability of plasmin to activate monocytes [10,11,14]. The levels of endotoxin in the plasmin stocks were determined by a dedicated commercial service. The highest experimental concentration of plasmin used resulted in the addition of ~0.05 ng/ml LPS to the cells. Treatment of RAW264.7 cells with an equivalent amount of an *E. coli* LPS was

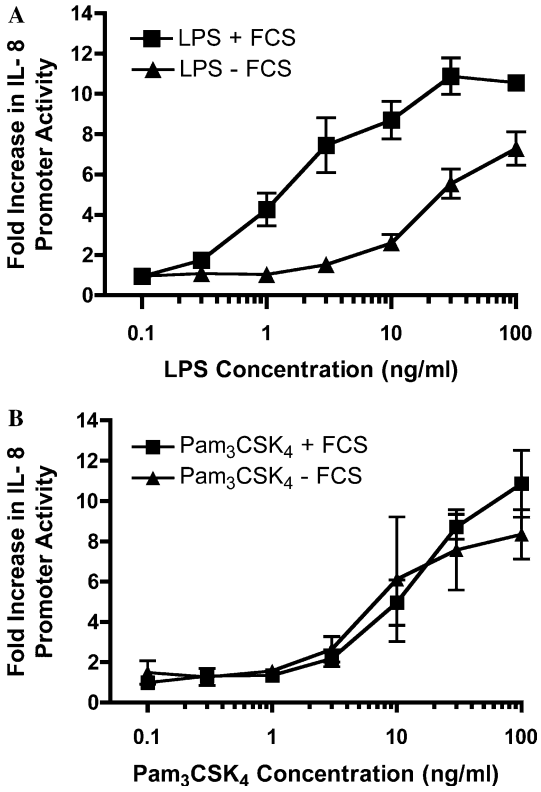

Fig. 1. Effects of serum on TLR4 and TLR2 responses. RAW264.7 cells were transfected with the NF-κB reporter system as described, and stimulated 24 h later with LPS or Pam_3CSK_4, in the presence or absence of 10% FCS, for a further 5 h. The cells were lysed and luciferase activity measured using a dual luciferase assay. Data were normalised for transfection efficiency as described, and effects of TLR agonists are presented relative to cells stimulated with media alone. (A) Data for cells activated with LPS, (B) for cells activated with Pam_3CSK_4. Data are $n = 3$ ±SEM.

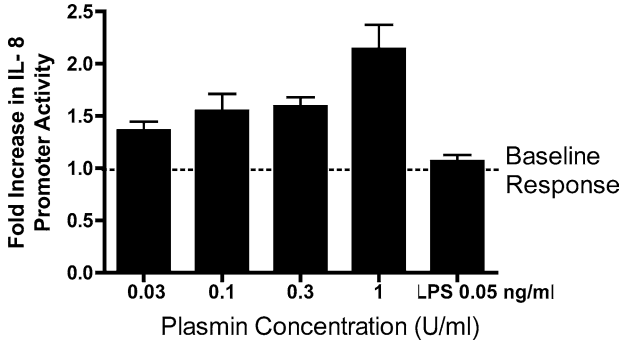

Fig. 2. Plasmin causes modest activation of an NF-κB reporter. RAW264.7 cells were transfected with the NF-κB reporter system as described and treated 24 h later with plasmin or LPS in the absence of serum (for 6 h). The cells were lysed and luciferase activity was measured using a dual luciferase assay. Data were normalised for transfection efficiency as described, and the effects of agonists are presented relative to cells stimulated with media alone. Data are $n = 3$ ±SEM.

insufficient to cause a similar activation of the NF-κB reporter (Fig. 2).

Plasmin pretreatment results in potentiation of TLR4 signalling

Having observed a direct activation of NF-κB when RAW cells were treated with plasmin, we investigated the potential for plasmin to enhance the response to LPS. Coincubation of plasmin and LPS for 5 h resulted in modest enhancement of LPS responses (data not shown). In further experiments, we pretreated RAW cells for 1 h with plasmin before adding LPS to the cells for a further 5 h (without removal of plasmin). Pretreatment with plasmin resulted in a marked increase in the luciferase generation observed in response to 10 ng/ml LPS (Fig. 3A). Similar actions of plasmin were seen using preparations from two manufacturers. These effects were not recreated by a similar pretreatment of RAW cells with a range of LPS concentrations (including amounts equivalent to the measured endotoxin contamination in the plasmin preparations). Plasmin pretreatment also potentiated responses to the TLR2 agonist, Pam_3CSK_4 (data not shown). Boiling (for 5 min) of plasmin prevented its ability to potentiate LPS responses (Fig. 3A), showing that a correctly folded protein structure was required for these actions. Additionally, protease inhibitors of plasmin (leupeptin and VPLK) inhibited the ability of plasmin to potentiate LPS signalling (this inhibition was significant for leupeptin, and just failed to reach statistical significance [$p = 0.07$] for VPLK, Figs. 3B and C). Plasmin pretreatment also significantly enhanced the generation of TNFα from untransfected RAW cells stimulated with LPS (Fig. 3D), an effect that was again inhibited by VPLK.

Protease inhibition does not directly inhibit LPS signalling

Protease inhibitors can inhibit LPS signalling by intracellular actions, preventing IκB breakdown [5,6]. In our studies, leupeptin and VPLK did not inhibit direct activation

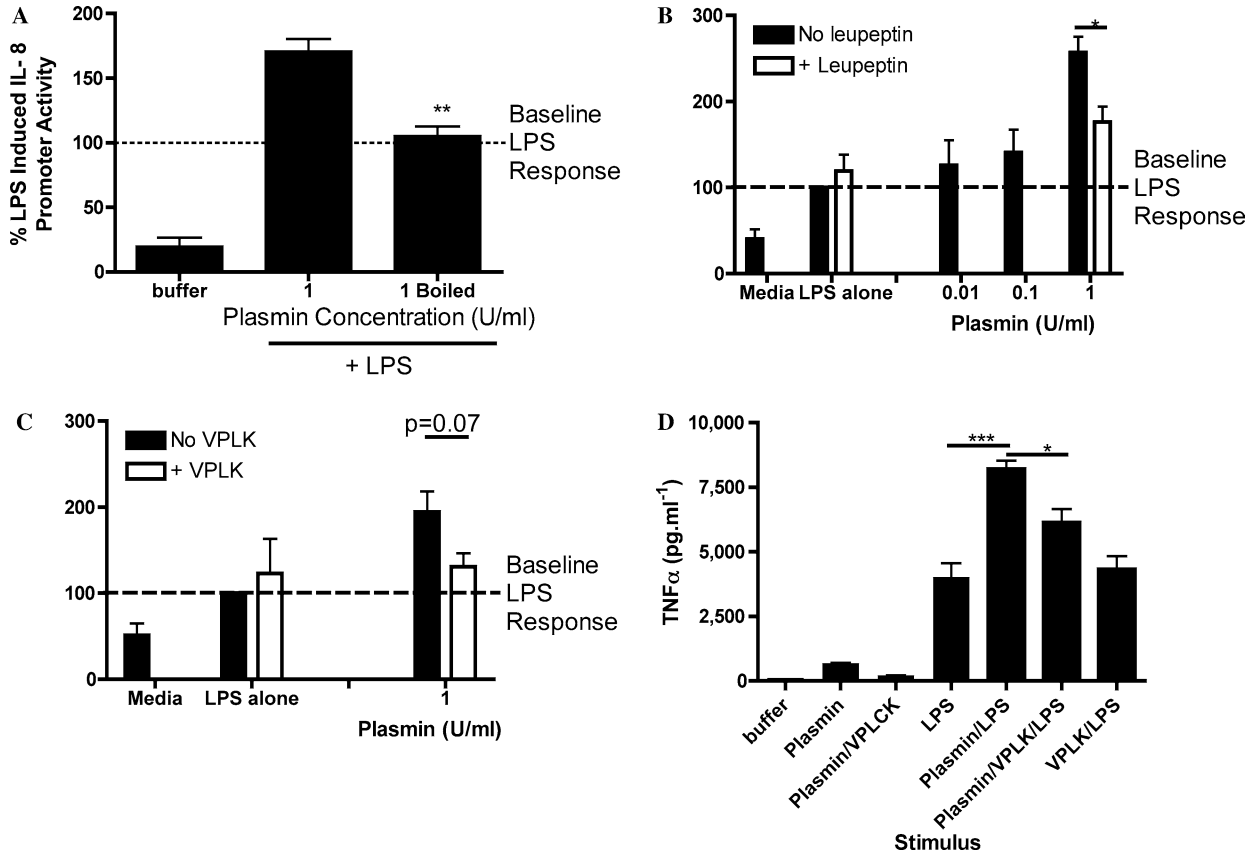

Fig. 3. Plasmin potentiates LPS responses. RAW264.7 cells were transfected with the NF-κB reporter system as described, and treated 24 h later with plasmin for one hour, followed by 10 ng/ml LPS (for 5 h) in the absence of serum. The cells were lysed and luciferase activity was measured using a dual luciferase assay. Data were normalised to the luciferase signal generated in response to LPS. (A) Plasmin pretreatment (1 U/ml) potentiated responses to LPS, and that these actions were abolished using boiled plasmin ($n = 3$ ±SEM, ** indicates suppression of plasmin effect by boiling, $p < 0.01$). (B,C) Inhibitors of plasmin were added to samples treated with LPS (1 ng/ml), or with 1 U/ml plasmin and LPS in combination (open bars). Fig. 3B shows plasmin potentiation of LPS responses was dose-dependent and inhibited by leupeptin ($n = 4$ ±SEM, $p < 0.05$ by paired t test). (C) Actions of plasmin were inhibited by VPLK ($n = 3$, $p = 0.07$ by paired t test). (D) Untransfected cells were stimulated with plasmin (1 U/ml) for 6 h (with or without VPLK) or with LPS (10 ng/ml) for 5 h after pretreatment with media or plasmin (1 U/ml) for 1 h. After stimulation, TNFα generation was measured. Plasmin enhanced LPS-induced TNFα generation, which was inhibited when plasmin was pretreated with VPLK. In contrast, VPLK did not inhibit LPS-induced TNFα generation in the absence of plasmin. * indicates $p < 0.05$ and *** indicates $p < 0.001$ by ANOVA and Tukey's post test.

of RAW cells by LPS (Fig. 3). These data are in keeping with work showing that VPLK did not inhibit LPS- and IL-1-induced monocyte activation [10], and suggest that their action was not on intracellular signalling pathways. Additionally, we did not observe inhibition of LPS responses in the presence of the serpin, antithrombin III (data not shown).

Plasmin potentiation of LPS signalling is not mediated by protease-activated receptors

Activation of protease-activated receptors (PARs) causes proinflammatory responses in a broad range of cell types. Monocytes express PAR1 and PAR2 [15], and plasmin may both activate [16] and inactivate PAR1 [17] in a dose-dependent fashion [18,19]. Once activated, PAR1 may transactivate PAR2 [20]. Thus, PAR activation presented an attractive mechanism by which plasmin might potentiate macrophage responses to LPS, but we were unable to demonstrate any ability of direct PAR1 and PAR2 agonistic peptides to potentiate LPS responses in a similar manner to that observed with plasmin (Fig. 4). Furthermore, thrombin, a potent agonist of PARs, was unable to potentiate LPS responses in experiments using doses of up to 10 U/ml when coincubated with LPS for 5 h (data not shown).

Discussion

The mechanisms regulating activation of TLR signalling at the cell surface remain challenging to determine [1]. Responses involve a series of molecular interactions between LPS and the proteins LBP, MD-2, and CD14, whose function is probably to monomerise LPS and overcome its hydrophobicity, allowing effective presentation to TLR4 [1]. Since serum contains potent antiprotease activity, we established whether the absence of serum would influence responses to LPS. We observed a reduced potency of LPS in the absence of serum, and thus our data support a role for serum proteins to maximise LPS responses in monocytic cells, however, serum proteins were not required to facilitate responses to the TLR2 agonist Pam_3CSK_4. Current evidence suggests that functional responses to individual TLR2 agonists in primary cells vary, perhaps dependent upon requirements of specific TLR2 agonists for CD14 signalling [21].

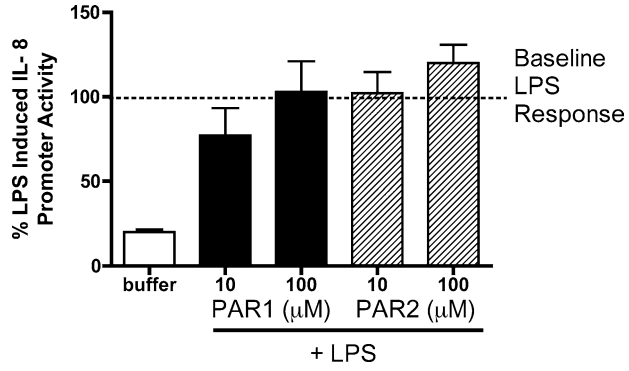

Fig. 4. Agonists of PAR1 and PAR2 do not potentiate LPS responses. RAW264.7 cells were transfected with the NF-κB reporter system as described, and treated 24 h later with media or PAR agonists for one hour, followed by media or 10 ng/ml LPS (for 5 h), in the absence of serum. The cells were lysed and luciferase activity was measured using a dual luciferase assay. Data were normalised to the luciferase signal generated in response to LPS. Open bars indicate luciferase activity of cells treated with media alone. PAR1 agonists (solid bars) and PAR2 agonists (hashed bars) were unable to prime LPS responses at the concentrations tested.

The dependence of LPS signalling on helper molecules, and difficulties in providing evidence of direct interaction of TLR agonists with their receptors, raises the possibility that other mechanisms may be involved in TLR activation. Even for TLRs where evidence of ligand interaction with the receptor has been demonstrated, the relevance of such interactions is not clear—for example, peptidoglycan binds to TLR2 [22], and was thought to be a TLR2 agonist, but it is now clear that peptidoglycan does not activate TLR2 but activates NOD-2 instead [23]. The complexity of TLR signalling led investigators to determine if parallels existed with the signalling systems of *Drosophila* Toll, where receptor activation depended on a proteolytic cascade. Our data, and that of other groups [6], do not support a role for proteolysis as an essential component of mammalian TLR activation. We have, however, revealed an ability of proteolytic cascades to potentiate LPS responses. Plasmin is a serine protease with important roles in the dissolution of fibrin clots and variable effects on PAR signalling [16–19]. Monocytes show significant expression of cell surface proteins that bind plasminogen and its activators, and plasmin generation is important in enabling transmigration through extracellular matrix [24]. We now describe an ability of plasmin to potentiate TLR2 and TLR4 responses in a macrophage cell line. A short phase of pretreatment prior to application of LPS (during which plasmin remained present) showed a synergistic signalling that was not replicated by PAR1 and PAR2 agonists, but which was dependent upon the catalytic activity of plasmin. These data are in keeping with other work showing proinflammatory actions of plasmin in monocytes [11]. Plasmin has an extensive range of protein targets and activities in monocyte biology [25]. The cellular mechanism that allows plasmin to cause potentiation of LPS responses is unknown, but it could be as a result of autocrine generation of proinflammatory mediators such as IL-1 and TNFα [10]. These data demonstrate a further important link between the regulation of coagulation and innate immunity, and provide new insights into the mechanisms resulting in vascular inflammation in response to microbial triggers.

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