The serine protease inhibitor antithrombin III inhibits LPS-mediated NF-κB activation by TLR-4

Ashley Mansell[a], Anna Reinicke[a,1], D. Margaret Worrall[b], Luke A.J. O'Neill[a,*]

[a] *Department of Biochemistry and Biotechnology Institute, Trinity College Dublin, Dublin 2, Ireland*
[b] *Department of Biochemistry, University College Dublin, Belfield, Dublin 4, Ireland*

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Abstract In *Drosophila*, the Toll family of proteins mediates the innate immune response. Toll is activated by Spaetzle, which is generated in response to pathogens via a serine protease cascade. We wished to investigate if lipopolysaccharides (LPS) might activate Toll-like receptor (TLR) 4 via a serine protease in humans. The serpin antithrombin III (ATIII) and the thrombin inhibitor hirudin both inhibited nuclear factor (NF)-κB activation by LPS and Lipid A. ATIII and hirudin were also able to inhibit LPS-induced NF-κB activation in cells stably transfected with TLR4. These results suggest that LPS may activate a mammalian serine protease, which generates a product required for TLR4 signalling. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Lipopolysaccharide; Inflammation; Signal transduction; Monocyte; Transcription factor

1. Introduction

Vertebrates have developed a sophisticated immune system to defend themselves against microbial infections, involving both innate and adaptive immune components. In *Drosophila* the Toll family of proteins mediates the innate immune response [1,2]. Toll-like receptors (TLR) also occur in humans, where 10 have been found [3]. Similar to *Drosophila* Toll, all of these possess a Toll/interleukin-1 (IL-1) receptor (TIR) cytoplasmic domain responsible for signal transduction [4,5]. Toll and TLRs activate Rel family transcription factors, which regulate host defense gene expression [6,7].

The role of Toll is to respond to fungal pathogens, while TLRs respond to different pathogen-derived products. Most notably, TLR4 responds to lipopolysaccharide (LPS) from Gram-negative bacteria [8,9], TLR2 responds to peptidoglycan from Gram-positive bacteria [10,11], TLR9 responds to bacterial CpG DNA [12], and TLR5 responds to bacterial flagellin [13]. TLRs have therefore been identified as so-called pathogen recognition receptors, which sense pathogen-associated molecular patterns (PAMPs). In spite of the similarities between the Toll system in *Drosophila* and mammals, there is evidence for a difference between them. In *Drosophila*, an extracellular serine protease cascade generates a proteolytically cleaved peptide, Spaetzle, the ligand for Toll, in response to fungi [14,15]. This cascade is inhibited by the serpin Spn43Ac [16]. In mammals, LPS has been proposed to act as the putative ligand for TLR4. The most convincing evidence for LPS binding to TLR4 has come from a cross-linking study in a cell line over-expressing TLR4 and two additional components needed for LPS signalling, MD2 and CD14. LPS was found to be cross-linked to all three [17].

Due to the fact that Toll action can be blocked by the serpin Spn43Ac in *Drosophila*, we wished to investigate whether the LPS response in mammals might also be sensitive to a serpin. We have found that acute treatment of the human monocyte cell line THP-1, or HEK 293 cells stably transfected with TLR4, with the serpin antithrombin III (ATIII), or the thrombin inhibitor hirudin, block nuclear factor (NF)-κB activation by LPS and Lipid A. These results provide the first indication that LPS may activate an extracellular serine protease in mammals, which is required for LPS-induced NF-κB activation.

2. Materials and methods

2.1. Cell culture

The human monocyte cell line THP-1 was purchased from European Collection of Cell Cultures (ECACC), and maintained in RPMI 1640 (Sigma, USA), supplemented with 10% foetal calf serum (FCS, Sigma, USA) and 2 mM L-glutamine (Gibco, UK), 5% CO_2. The stably transfected cell line HEK 293-TLR4 was a kind gift from Prof. Douglas Golenbock (Boston Medical Centre, Boston, MA, USA), and were maintained in RPMI 1640, 10% FCS, 2 mM L-glutamine, 5% CO_2 supplemented with selection antibiotic 25 μg/ml puromycin.

2.2. Materials

Chromozym TH was supplied by Boehringer Mannheim (Mannheim, Germany). The α-isoform of both ATIII and the latent ATIII were a kind gift of Prof. Robin Carrell (Cambridge University, UK). Recombinant leech hirudin [Lys^{47}]rHV2 variant, LPS serotype 026:B6 and Lipid A were supplied by Sigma. The 22-bp oligonucleotide, containing the NF-κB consensus sequence and T4 polynucleotide kinase kit were from Promega Corp (WI, USA). [γ-^{32}P]ATP (3000 Ci/mol) is from Amersham

2.3. Nuclear extract preparation and electrophoretic mobility shift assays (EMSA)

Assays were conducted as previously described [18]. Briefly, THP-1 cells were resuspended in serum-free RPMI 1640 at 1×10^6/ml. Cells were pre-treated with relevant concentrations of proteins for 1 min,

*Corresponding author.
E-mail address: laneill@tcd.ie (L.A.J. O'Neill).

[1] Present address: Department of Pathology, Tennis Court Rd, University of Cambridge, Cambridge CB2 1QP, UK.

Abbreviations: TLR, Toll-like receptor; ATIII, antithrombin III; L-ATIII, latent antithrombin III; LPS, lipopolysaccharide; EMSA, electrophoretic mobility shift assay; IL-1, interleukin-1; TNF, tumour necrosis factor

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stimulated with LPS as before, and incubated for a further 60 min at 37°C. Experiments using stably transfected HEK 293-TLR4 cells were performed as above, however, cells were seeded at 5×10^4/ml, 48 h prior to stimulation. Media were replaced with serum-free RPMI prior to simulation. Nuclear extracts were prepared by replacement of media from cells with ice-cold hypotonic buffer. The subsequent cell pellet was lysed and placed on ice for 10 min. Nuclear-associated proteins were extracted and maintained on ice for 20 min. Following centrifugation, the supernatant was mixed with storage buffer and used immediately, or frozen at −20°C. Protein concentrations were determined using the method of Bradford [19] and extracts stored at −20°C. In the EMSA, nuclear extracts were incubated for 30 min with 10 000 cpm of a 22-bp DNA fragment oligonucleotide containing the NF-κB consensus sequence previously been labelled with [γ-^{32}P]ATP, in the presence of poly(dI.dC) and 10× binding buffer. Incubated mixtures were subjected to electrophoresis on native 5% (w/v) polyacrylamide gels, which were subsequently dried and autoradiographed. Poly(dI.dC) was supplied by Pharmacia (Uppsala, Sweden).

2.4. Protease assay

THP-1 cells (7.5×10^4/ml) were resuspended in freshly prepared RPMI 1640 (phenol-red-free) containing 2% FCS in 96 well plates. Cells were stimulated with 10 μg/ml of LPS for 3 min. 30-μl samples were removed and diluted into 170 μl of Chromozym TH (1.25 mg/ml) dissolved in phenol-red-free RPMI 1640, 2 mM L-glutamine. Chromozym TH is a tosyl-glycyl-prolyl-arginine-4 nitranilide acetate substrate, with specificity for 'thrombin-like' proteases. Absorbance readings were taken at the indicated time points. Results are displayed as arbitrary absorbance units, displayed as increase in absorbance units in regard to time zero, per time point.

2.4.1. Statistical analysis. Significance was evaluated using the Student's *t*-test for unpaired data.

3. Results

3.1. ATIII and hirudin inhibit LPS-mediated NF-κB activation

We first investigated whether the serpin, ATIII, a plasma inhibitor of thrombin-like proteases, could inhibit LPS-mediated NF-κB activation [20]. As shown in Fig. 1, ATIII was effective in reducing LPS-mediated NF-κB activation if cells were pre-treated for no more than 1–5 min. Simultaneous addition of ATIII and LPS had no effect, while longer pre-treatments were found to be ineffective in reducing NF-κB activation. The inhibitor pre-treatment time is therefore critical for an inhibitory effect to be observed. This is presumably due to endogenous serine protease activity of the cells, which would inactivate the ATIII in longer pre-treatment times. The failure of co-incubation by ATIII to inhibit suggests that the activation of the protease by LPS is more rapid than the inhibitory effect of ATIII.

Using a pre-treatment time of 1 min, we next pre-treated cells with a range of concentrations of ATIII. As can be seen in Fig. 2A, while ATIII was able to inhibit LPS-mediated NF-κB activation over a broad concentration range of 0.5–20 μg/ml, optimal inhibition was achieved with 10 μg/ml.

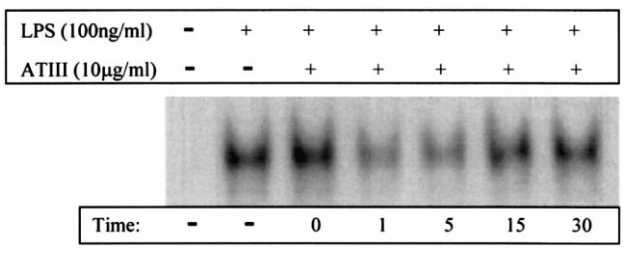

Fig. 1. Pre-treatment time with ATIII is crucial in mediating inhibitory effect on NF-κB activation by LPS. THP-1 cells (1×10^6/ml) were resuspended in serum-free media, and pre-treated with 10 μg/ml ATIII for indicated times. Cells were stimulated with LPS (100 ng/ml) and incubated for a further 60 min. Nuclear extracts were prepared and assayed for NF-κB activation by EMSA. Identical results were obtained in two further experiments.

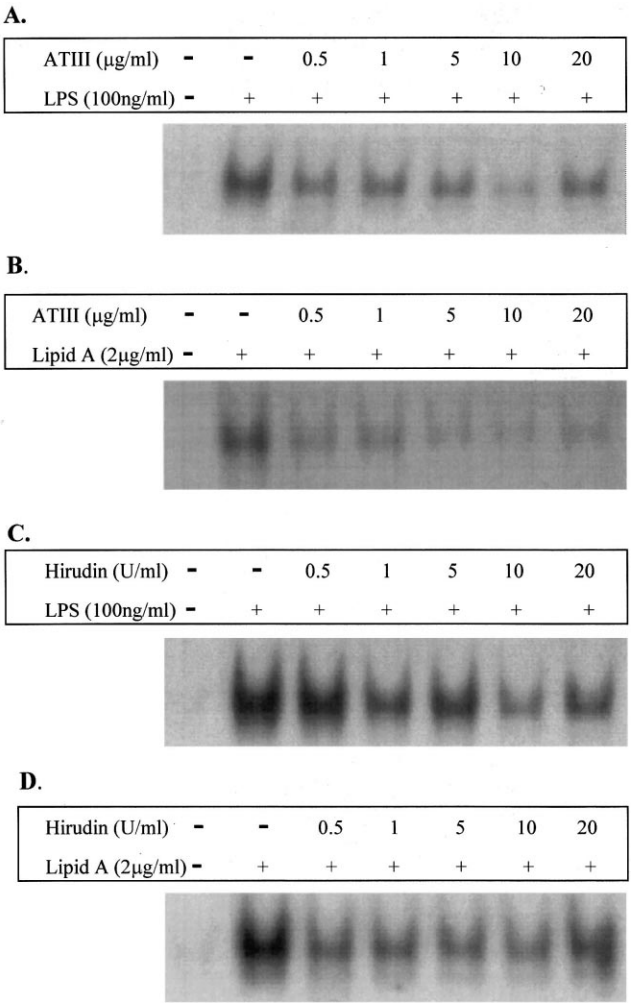

Fig. 2. ATIII and hirudin inhibit both LPS- and Lipid A-mediated NF-κB activation. THP-1 cells (1×10^6/ml) were resuspended in serum-free media, and pre-treated with 0.5–20 μg/ml ATIII (A, B) or 0.5–20 units/ml hirudin (C, D) for 1 min. Cells were stimulated with either 100 ng/ml LPS (A, C) or 2 μg/ml Lipid A (B, D) and incubated for a further 60 min. Nuclear extracts prepared and assayed for NF-κB activation by EMSA. Identical results were obtained in a further experiment.

In plasma, ATIII binds heparin, thereby increasing its inhibitory effect against thrombin [21]. We found however, that heparin had no effect on either inhibitory or sub-inhibitory levels of ATIII and its ability to inhibit LPS-mediated activation of NF-κB (data not shown).

To further examine the effect of ATIII, we also tested ATIII against Lipid A, the synthetic active moiety of LPS [22]. Lipid A does not contain lipoproteins or other contaminants that have previously been shown to induce NF-κB activation [23]. Fig. 2B clearly illustrates the ability of ATIII to abrogate Lipid A-induced NF-κB activation over the same concentration range as that observed for LPS.

We next wished to determine if hirudin, another thrombin inhibitor [24], could inhibit LPS-mediated NF-κB activation. As shown in Fig. 2, hirudin inhibited NF-κB activation by

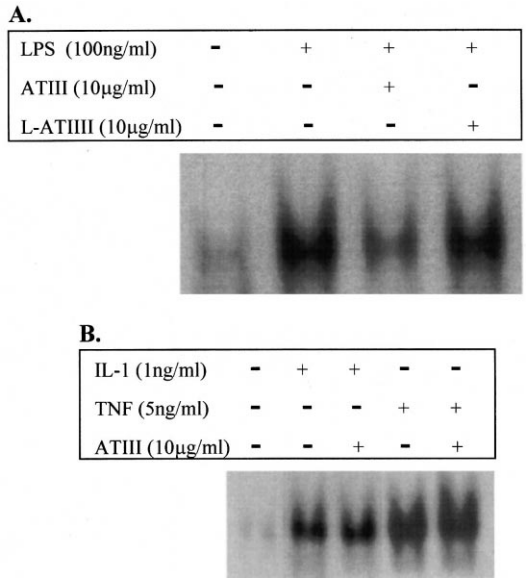

Fig. 3. Specificity of ATIII inhibitory effect. A: THP-1 cells (1×10^6/ml) were resuspended in serum-free media, and pre-treated for 1 min with either 10 μg/ml ATIII or 10 μg/ml L-ATIII. Cells were stimulated with 100 ng/ml LPS and incubated for a further 60 min. Nuclear extracts prepared and assayed for NF-κB activation by EMSA. Similar results were obtained in a further experiment. B: THP-1 cells (1×10^6/ml) were resuspended in serum-free media, and then indicated cells pre-treated with 10 μg/ml ATIII for 1 min. Cells were then stimulated with either 5 ng/ml TNFα or 1 ng/ml IL-1 and incubated for a further 60 min. Nuclear extracts prepared and assayed for NF-κB activation by EMSA. Similar results were obtained in a further experiment.

both LPS (Fig. 2C) and Lipid A (Fig. 2D), over a concentration range of 1–20 units/ml.

Thrombin has previously been shown to activate NF-κB [25]. We wished to determine if thrombin was responsible for the effect observed here. Treatment of THP-1 cells with thrombin (10–1000 μg/ml), failed to induce activation of NF-κB (data not shown). It has also previously been shown that thrombin is able to potentiate LPS activation of cells [26]. We found however, that thrombin failed to potentiate NF-κB activation at sub-inducible LPS concentrations combined with a range of thrombin concentrations (10–1000 μg/ml) (data not shown).

Taken together, these results would suggest that LPS activation of NF-κB requires the activation of a protease that is inhibited by ATIII and hirudin, but importantly, does not involve free thrombin.

3.2. ATIII inhibition is specific for LPS-induced NF-κB activation

We next confirmed that the effect of ATIII requires its serpin activity. Cells were pre-treated with both ATIII and an inactive latent form of ATIII (L-ATIII), the inhibitory loop of which is inserted into the A-β sheet and is thus inaccessible to proteases [27]. Fig. 3 demonstrates that L-ATIII (10 μg/ml) was unable to inhibit LPS-mediated NF-κB activation. Higher concentrations of L-ATIII also had no effect (data not shown).

We also tested the specificity of ATIII with respect to LPS, by testing the effect of two other NF-κB activators, IL-1 and tumour necrosis factor (TNF)-α.

These two cytokines have previously been shown to activate NF-κB [28], and in the case of IL-1, require a similar signal transduction pathway as LPS to induce NF-κB nuclear translocation [4–7]. As shown in Fig. 3B, ATIII was unable to inhibit NF-κB activation induced by either IL-1 or TNFα. This result would suggest that the inhibitory effect of ATIII is not affecting common signal transduction events to NF-κB within the cytosol.

3.3. ATIII and hirudin inhibit LPS-mediated NF-κB activation via TLR4

We also tested the effect of ATIII on NF-κB activation by LPS in HEK 293 cells stably transfected with TLR4. Parental 293 cells do not respond to LPS (not shown). As can be seen in Fig. 4, ATIII was able to inhibit LPS-mediated NF-κB activation. Hirudin at 10 units/ml was also able to reduce this effect. However, NF-κB activation by LPS was unaffected by L-ATIII (10 μg/ml).

3.4. LPS induces activation of a thrombin-like protease

We next wished to investigate whether LPS could induce the rapid activation of a serine protease in mammalian cells. THP-1 cells were therefore treated with LPS and protease activity monitored using a substrate that is specifically cleaved by thrombin-like proteases. As demonstrated in Fig. 5, while THP-1 cells demonstrate considerable basal protease activity in non-stimulated cells, there is an increase in protease activity observed within 7.5 min post-stimulation. This protease activity was significantly enhanced above non-stimulated basal activity between 15 and 30 min post-LPS treatment. By 60 min no difference between untreated and stimulated cells was observed. These results suggest that LPS can induce a thrombin-like protease activity over basal activity, in a time-dependent manner in THP-1 cells.

4. Discussion

In *Drosophila*, Toll responds to fungal pathogens [1,2]. Genetic evidence suggests that a serine protease cascade generates Spaetzle, the putative ligand of Toll [14] (although there are no direct binding studies). Additional data on the possibility of a serine protease cascade has been obtained. Loss of function mutation of the serpin, Spc43Ac, leads to constitutive Spaetzle and Toll activation [14–16]. Though mammalian

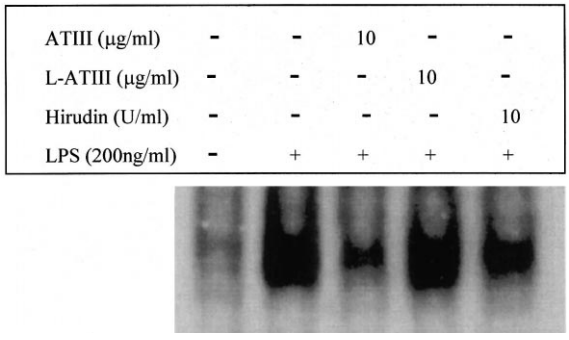

Fig. 4. ATIII and hirudin inhibit LPS-induced NF-κB activation by TLR4. Stably transfected HEK 293-TLR4 cells (5×10^4/ml) were seeded 48 h prior to treatment. Cells were pre-treated for 1 min with indicated serine protease inhibitor, then stimulated with 200 μg/ml LPS and incubated for a further 60 min. Nuclear extracts were prepared and assayed for NF-κB activation by EMSA. Similar results were obtained in a further experiment.

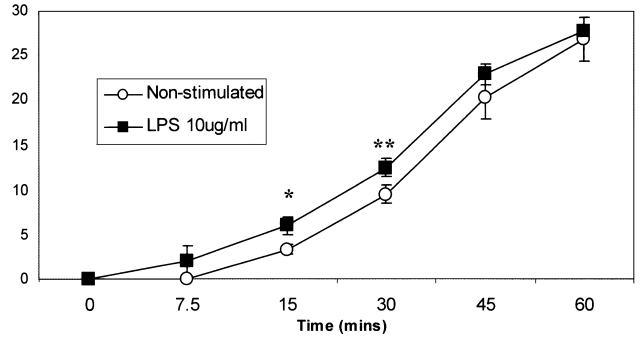

Fig. 5. LPS induce thrombin-like protease activity. THP-1 cells (7.5×10^4/ml) were stimulated with 10 μg/ml of LPS. Samples were diluted in phenol-red-free RPMI containing Chromozym TH and spectrometrically assayed at relevant time points. Results are shown as absorbance units per individual sample and are expressed as means ± standard deviations of triplicate samples from three experiments. Data indicate significant differences ($*P=0.016$ and $**P=0.021$) when compared with non-stimulated control values.

TLR4 has been described as the receptor for LPS [8,9], no direct binding studies have been performed and the affinity of the complex for LPS has not been determined. Prompted by this study in *Drosophila* on Spc43Ac, we tested the effect of the serpin ATIII on NF-κB activation by LPS in the human monocyte cell line THP-1. ATIII has been shown to protect animals from LPS-induced septic shock and also been shown to inhibit induction of IL-6 (a NF-κB-dependent gene) by LPS.

We found that ATIII was able to specifically inhibit NF-κB activation induced by LPS over a range of concentrations. Crucially, the effect of ATIII was time-dependent. The protease activity assay we carried out suggested a high basal thrombin-like protease activity. ATIII would presumably be inactivated by the endogenous activity, limiting its efficacy. Co-incubation of ATIII with LPS did not result in inhibition, suggesting that the LPS-activated protease proceeds at a faster rate than ATIII inhibition.

The effect of ATIII required its serpin activity since an inactive form of ATIII was unable to inhibit LPS-mediated NF-κB activation. ATIII was able to reduce LPS-induced activation of NF-κB by TLR4. This result suggests the possibility that a protease generates a product that is able to activate TLR4. It is possible that the protease being activated is thrombin, since hirudin, whose only known target is thrombin, also had an inhibitory effect. However, heparin did not enhance the inhibitory effect of ATIII and thrombin was unable to activate NF-κB in the cells. It is possible hirudin is targeting membrane-associated thrombin which may not be mimicked by the addition of free thrombin. We found that LPS could activate a thrombin-like protease in THP-1 cells. The activity was transient in nature, and required above normal concentrations of LPS. The effect was subtle but consistent, which would be expected if LPS were activating a specific serine protease, generating a specific ligand.

Though no explicit evidence has been shown supporting direct binding between LPS and TLR4, two studies have been published suggesting such a direct interaction [17,29]. Lipid IVa, a partial structure of the active LPS moiety Lipid A, acts as an agonist on mouse TLR4, but is an antagonist in humans. Transfection of mouse macrophages with human TLR4 conferred an antagonist effect to Lipid IVa, while human macrophages transfected with mouse TLR4, detected Lipid IVa as an agonist [29]. These findings suggest that TLR4 alone is able to determine the response to Lipid IVa. Other data suggests that MD2 is responsible for this species-specific pharmacology [30]. In the absence of direct binding data, it is difficult to evaluate these data. While it is likely that TLR4 and MD2 are involved in LPS recognition, anti-TLR4 antibodies that inhibit LPS-induced NF-κB-dependent gene expression were unable to reduce radiolabelling of TLR4 and MD2 complex [17]. This suggests that additional steps are required for cell activation, besides the close proximity of LPS to TLR4 and MD2. The authors suggest that the antibody interferes with oligomerisation. Another possibility is that the antibody blocks the binding of an endogenous ligand. The clear close proximity of LPS to CD14, MD2 and TLR4 does not exclude the possibility of a further protein binding LPS and triggering a serine protease cascade, which results in a ligand for TLR4.

In conclusion, our data suggest that the rapid activation of a serine protease is required for TLR4 function. It is possible that TLR4 binding is required for protease activation, the resulting ligand acting via TLR4, in an analogous manner to Spaetzle and Toll in *Drosophila*. Further studies to identify the protease involved are underway.

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