Elafin prevents lipopolysaccharide-induced AP-1 and NF-kappaB activation via an effect on the ubiquitin-proteasome pathway.

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ELAFIN PREVENTS LIPOPOLYSACCHARIDE-INDUCED AP-1 AND NF-κB ACTIVATION VIA AN EFFECT ON THE UBIQUITIN-PROTEASOME PATHWAY

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Running Title: Elafin modulation of LPS signalling in monocytes

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The serine anti-protease elafin is expressed by monocytes, alveolar macrophages, neutrophils and at mucosal surfaces, and possesses antimicrobial activity. It is also known to reduce lipopolysaccharide (LPS)-induced neutrophil influx into murine alveoli as well as abrogating LPS-induced production of MMP-9, MIP-2 and TNF-α by as yet unidentified mechanisms. In this report we have shown that elafin inhibits the LPS-induced production of MCP-1 in monocytes by inhibiting AP-1 and NF-κB activation. Elafin prevented LPS-induced phosphorylation of ATF2, c-jun and JNK, but had no effect on LPS-induced phosphorylation of p38. The LPS-induced degradation of IRAK, IκBα and IκBβ was inhibited by elafin, but phosphorylation of IκBα was unaffected. Polyubiquitinated protein was shown to accumulate in the presence of elafin. These results suggest that elafin’s inhibition of LPS-induced AP-1 and NF-κB activation occurs via an effect on the ubiquitin-proteasome pathway.

Elafin is a 6 kilodalton serine anti-protease initially identified in psoriatic skin scales, and also called skin-derived anti-leucoprotease (SKALP). Subsequently, “elastase-specific inhibitor” was isolated from human sputum, and found to have identical N-terminal sequencing as SKALP, and the term elafin was suggested (1). Elafin is currently known to be expressed in airways, other mucosal surfaces such as oesophagus, vagina, endometrium, coronary intima, and by inflammatory cells such as monocytes, alveolar macrophages and neutrophils.

Structurally, elafin is a member of the WAP (whey acidic protein) family of proteins characterised by possessing a C-terminal core domain consisting of 4 disulphide bonds. Also known as trappsins (TRansglutaminase substrate and wAP domain containing ProteIN), these proteins also possess an N-terminal domain consisting of a variable number of repeats with the consensus sequence Gly-Gln-Asp-Pro-Val-Lys that can act as an anchoring motif by transglutaminase cross-linking (2). Pre-elafin, also known as trappin 2, is thought to undergo proteolytic cleavage, possibly by tryptase, releasing the elafin molecule (3). Elafin is a cationic protease inhibitor of human neutrophil elastase (HNE), proteinase 3 and porcine pancreatic elastase, and is induced by cytokine and other stimuli including IL-1β, TNF, HNE and lipopolysaccharide (LPS) (1). The gene governing elafin (and other WAP proteins) expression was cloned and sequenced on the long arm of chromosome 20. Transcriptional activation of elafin appears to be cell-specific; in pulmonary epithelial cells elafin is regulated at a transcriptional level by the transcription factor nuclear factor kappa B (NF-κB) (4) while in mammary epithelial cells the transcription...
factor activating protein-1 (AP-1) mediates transcriptional activation (5).

The observation that elafin is highly cationic and shows selective expression at mucosal, epithelial and endothelial surfaces suggested that it possesses antimicrobial properties. Indeed, elafin has been shown to have antimicrobial activity which is independent of its anti-elastase activity or charge properties (6). In mice, pre-elafin has been shown to dose-dependently reduce LPS-induced neutrophil influx into alveoli, in addition to inhibiting LPS-induced production of the potent neutrophil attractants MMP-9 (gelatinase) and MIP-2 (macrophage inflammatory protein-2), suggesting an immunomodulatory role in innate immunity (7). We have previously shown that secretory leucoprotease inhibitor (SLPI), another WAP protein closely related to elafin, prevents lipopolysaccharide-induced IκBα degradation without affecting phosphorylation or ubiquitination in monocytic cells (8), and furthermore, that SLPI can also impair lipotechoic acid (LTA)-induced proinflammatory gene expression in monocytes and macrophages in vitro, processes diminished by oxidation and elastase complex formation of SLPI (9).

In light of these results, we have looked at the modulatory effects of elafin on LPS signalling in U937 cells, including elafin’s effect on LPS-induced monocyte chemotactic protein-1 (MCP-1). MCP-1 is a CC chemokine produced by mononuclear phagocytes and is a potent activator of monocyte function (10,11). MCP-1 has a crucial role in monocyte recruitment in vivo in several organs and tissues (12-14) and its role in monocyte infiltration in several inflammatory diseases has been described (10,11). MCP-1 gene expression is cell-type and stimulus-specific, and its transcription is regulated by transcription factors including AP-1 and NF-κB (15-18). In addition, LPS-induced MCP-1 expression has been shown to be dependent on c-jun NH2-terminal kinase (JNK) activation (19). In this report we show that elafin inhibits the LPS-induced production of MCP-1 in U937 cells via inhibition of AP-1 and NF-κB, and that this occurs through elafin’s effect upon the ubiquitin-proteasome pathway.

Experimental Procedures

Materials— RPMI 1640 medium was obtained from GIBCO, and U937 cells were purchased from the American Type Culture Collection (Manassas, VA). Recombinant human Elafin was obtained from Proteo Biotech AG (Kiel, Germany). Antibodies to phosphorylated and total ATF2, c-jun, JNK and IκBα, and to IRAK, IκBβ and phosphorylated p38 were obtained from Cell Signaling Technology (Beverly, MA). Antibody to p38 and lamin B1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). IRAK-1 antibody was obtained from BD Biosciences (San Jose, CA). Antibody to ubiquitin was purchased from Sigma. Western blotting reagents were obtained from Pierce Biotechnology (Rockford, IL). Fluorogenic substrates for 20 S proteasome activity assays were purchased from Merck Biosciences (Nottingham, UK).

Cell Culture— Human myelomonocytic U937 cells were cultured in RPMI 1640 containing 10% fetal calf serum, 2 mM glutamine, penicillin, and streptomycin and were maintained at 37 °C in a humidified atmosphere of 5% CO2.

Preparation of Cytoplasmic and Nuclear Extracts— After treating cells with elafin and/or LPS for the indicated times in 24-well plates (1 x 106 cells/ml), the cells were washed in ice-cold phosphate-buffered saline and resuspended in a hypotonic buffer (10mM HEPES, pH 7.9, 1.5mM MgCl2, 10mM KCl, and 0.5 mM DTT). Cells were pelleted by centrifugation at 14000 x g for 10 min at
4°C and then lysed for 10 min on ice in 20µl of hypotonic buffer containing 0.1% Igepal CA-630, 1mM sodium vanadate, 50mM β-glycerophosphate and 1 x complete protease inhibitor mixture (Roche Molecular Biochemicals). Lysates were centrifuged as before, and the supernatant (cytoplasmic fraction) was retained for Western analysis (see below). The remaining nuclear pellet was lysed in 15µl of lysis buffer (20mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 1mM sodium vanadate, 50mM β-glycerophosphate and 1 x complete protease inhibitor mixture) for 15 min on ice. After centrifugation at 14,000 × g for 15 min at 4 °C, nuclear extracts were removed, and the extracts were stored at −80 °C.

EMSA—Nuclear extracts (5 µg) were incubated with biotin end-labeled double-stranded oligonucleotide containing the AP-1 or NF-κB consensus sequences (MWG Biotech, Germany). Incubations were performed for 30 min at room temperature in binding buffer (4% (v/v) glycerol, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM DTT, 0.1 mg/ml nuclease-free bovine serum albumin) and 2 µg of poly(dI·dC) (Sigma). Reaction mixtures were electrophoresed on native 6% polyacrylamide gels and blotted. Transferred nuclear proteins were cross-linked by UV transillumination, blocked, incubated with streptavidin peroxidase and finally developed by chemiluminescent methodology (Pierce Biotechnology, Rockford, IL).

Immunochemical Detection of Proteins--After treating cells with the indicated reagents for the indicated times cytoplasmic extracts were prepared as shown above. Protein concentration of the extracts was determined using the Bradford method (20). Equal amounts of protein from each sample (20 µg for each of phosphorylated ATF2 and phosphorylated c-jun, and 40 µg for each of phosphorylated JNK, phosphorylated p38, IRAK, IκBα, IκBβ, phosphorylated IκBα and ubiquitin) were electrophoresed by SDS-PAGE and blotted. Transferred proteins were blocked in 5% dried skimmed milk and 1% bovine serum albumin in PBST for all proteins except phosphorylated JNK, where 5% bovine serum albumin in PBST was used. Proteins were detected using primary antibodies directed against total and phosphorylated ATF2, c-jun, JNK, phosphorylated p38, IκBα, IκBβ and phosphorylated IκBα (1:1000), p38, lamin B1, IRAK, and ubiquitin (1:200) and phosphorylated p38 (1:1000) followed by incubation with horse radish peroxidase (HRP)-conjugated secondary antibodies (1:2000). Antigen-antibody complexes were detected with enhanced chemiluminescence reagents (Pierce Biotechnology, Rockford, IL).

20 S Proteasome Activity Assays- Three different peptidase activities associated with the 20 S proteasome were measured using the fluorogenic substrates Suc-Leu-Leu-Val-Tyr-AMC (for chymotrypsin-like activity), Z-Leu-Leu-Glu-AMC (for peptidylglutamyl peptide hydrolizing activity), and Z-Ala-Arg-Arg-AMC (for trypsin-like activity). Treated cells were lysed in 700 µL of 25mM HEPES, 5mM EDTA, 0.1% CHAPS, 5mM ATP, pH 7.5, with 2mM DTT (21). Each sample was incubated with each substrate (50 µM, final concentration) in lysis buffer for 30 mins at 37 °C, and fluorescence (substrate turnover) was determined by excitation at 355 nm and emission at 460 nm.

RESULTS

U937 cells were incubated with LPS for 24 h following time course and dose-response experiments (data not shown), and some samples were preincubated with elafin (10µg/ml) for 1 h followed by incubation with LPS. LPS was found to induce significantly more MCP-1 production in U937 cells compared to cells incubated in medium alone. Elafin inhibited LPS-induced MCP-1 production (Fig. 1A). Elafin incubation alone did not
alter basal MCP-1 levels. These experiments were repeated three times with similar results.

Because MCP-1 expression is regulated by the transcription factors, AP-1 and NF-κB, the effect of elafin on LPS-induced transcription factor activation was examined by pre-incubating U937 cells with elafin (1 h) followed by LPS over the time courses shown (Fig. 1B and 1C). LPS was shown to induce significantly more AP-1 activation in U937 cells (Fig. 1B, left, lanes 30, 60 and 120) compared to cells incubated in medium alone (Fig. 1B, left, lane Con). Elafin prevented LPS-induced AP-1 activation across the time course (Fig. 1B, right). Similarly, LPS induced significantly more NF-κB activation (Fig. 1C, left, lanes 15, 30 and 60) versus control (Fig. 1C, lane Con), which was markedly inhibited by elafin (Fig. 1C, right).

To investigate the effect of elafin on LPS-induced phosphorylation of AP-1 subunits, phosphorylation of activating transcription factor 2 (ATF2) and c-jun was assessed. LPS was shown to induce phosphorylation of ATF2 (Fig. 2A, top panel, left), and this phosphorylation was significantly abrogated by pre-incubation with elafin (Fig. 2A, upper panel, right). Protein loading was controlled for by probing the stripped blot for total ATF2 (Fig. 2B, lower panel). Similarly, LPS-induced phosphorylation of c-jun was prevented by pre-incubation with elafin (Fig. 2B, upper panel). Again, protein loading was controlled for by stripping the blot and reprobing for total c-jun (Fig. 2B, lower panel).

ATF2 and c-jun are activated by kinases including the mitogen-activated protein (MAP) kinases p38 and JNK respectively. To assess the effect of elafin on LPS-induced phosphorylation of p38, cells were again either incubated with LPS alone or pre-incubated with elafin followed by LPS in a time course experiment. Equal amounts of cytoplasmic extract were electrophoresed, blotted and incubated with anti-phosphorylated p38. Elafin had no effect on the LPS-induced phosphorylation of p38 (Fig. 3A, upper panel). To confirm this lack of an effect, the stripped blots were reprobed for total p38, which showed equal loading (Fig. 3A, lower panel). Nuclear extracts from the same experiments were similarly probed for phosphorylated and total p38, and again, elafin was shown to have no effect on the LPS-induced phosphorylation of p38 (Fig. 3B). In order to investigate the effect of elafin on LPS-induced phosphorylation of JNK, U937s were either incubated with LPS or pre-incubated with elafin followed by LPS over a time course of 0, 15, 30, 60 and 120 min. Elafin prevented the LPS-induced phosphorylation of JNK in cytoplasmic extracts analyzed by Western blot (Fig. 3C, upper panel), with protein loading controlled for by reprobing the stripped blots for total JNK (Fig. 3C, lower panel). Similarly, the nuclear extracts from these experiments were subjected to Western analysis and again, elafin prevented the LPS-induced phosphorylation of JNK, with lamin B1 shown as a loading control (Fig. 3D).

To assess the effect of elafin on NF-κB regulatory proteins, degradation of IL-1R-associated kinase (IRAK), IκBα and IκBβ were assessed. U937 cells were again either incubated with medium alone, with LPS or pre-incubated with elafin followed by LPS over the time courses shown (Fig. 4). Western blot analyses of cytoplasmic extracts showed LPS-induced degradation of IκBα, IκBβ and IRAK (Fig. 4 A-C, left rows), whereas preincubation with elafin resulted in inhibition of the degradation of these proteins (Fig. 4 A-C, right rows).

To investigate whether elafin affects the phosphorylation of IκBα, cells were treated with or without the proteasome inhibitor ALLN (100 µg/ml for 30 mins), used to stabilize the labile phosphorylated IκBα. The U937s were incubated in medium alone, medium with ALLN, stimulated with LPS in the presence of ALLN, or preincubated with ALLN and then elafin followed by stimulation with
LPS. Phosphorylation of IκBα was observed in all samples treated with ALLN (Fig. 5A, lanes 3-8), though only weakly so in the samples not also treated with LPS (Fig. 5A, lanes 3-4). The increased phosphorylation of IκBα seen in the samples pretreated with elafin (Fig. 5A, lanes 7-8) was similar to that observed in the samples treated with ALLN and LPS alone (Fig. 5A, lanes 5-6).

Because the NF-κB regulatory proteins IRAK, IκBα and IκBβ undergo ubiquitination in response to stimuli such as LPS, and because elafin did not appear to prevent the LPS-induced phosphorylation of IκBα, we assessed the possible effect of elafin on ubiquitin. U937 cells were incubated with medium alone, or stimulated with LPS with or without preincubation with elafin and cytoplasmic lysates were obtained. LPS-induced polyubiquitination appeared to increase in the prior presence of elafin (Fig. 5B, right lanes) compared to when stimulated with LPS alone (Fig. 5B, left lanes), as assessed by Western blot analysis.

DISCUSSION

Elafin inhibits LPS-induced production of MCP-1 in the U937 monocytic cell line by preventing the LPS-induced activation of AP-1 and NF-κB. This anti-inflammatory effect appears to be dependent upon an effect of elafin on the ubiquitin-proteasome pathway.

LPS activates the transcription factors AP-1 and NF-κB via a process that initially involves binding of LPS to the cell membrane proteins, TLR4 and MD-2, which is facilitated by CD14 (22). Signal can then be transduced via a MyD88-dependent pathway involving IRAK and TNF-receptor associated factor (TRAF)-6 resulting in activation of the MAPK kinase, TAK1 (23,24). From this point, TAK-1 acts as a common activator of NF-κB and AP-1 pathways. The subsequent phosphorylation of a group of inhibitory-binding proteins kappa B (IκB) allows them to be ubiquitinated and degraded, which allows NF-κB to translocate into the nucleus (25). In the AP-1 pathway, JNK phosphorylation leads to activation of a number of proteins including ATF-2 and c-jun, subunits of AP-1 (26).

In our study, elafin prevented the LPS-induced phosphorylation of JNK and the subsequent phosphorylation of the AP-1 subunits, ATF2 and c-jun. Although the kinase p38 is also responsible for the phosphorylation and activation of AFT2, elafin had no effect on the LPS-induced phosphorylation of p38. In addition, elafin also prevented the LPS-induced degradation of the NF-κB regulatory proteins, IRAK, IκBα and IκBβ. These proteins are regulated by phosphorylation, ubiquitination and proteasomal degradation (25). While elafin appeared to have no effect on LPS-induced phosphorylation of IκBα, we found that elafin appears to delay ubiquitination of proteins in response to LPS, which corresponded to the inhibition of LPS-induced IRAK, IκBα and IκBβ degradation in the presence of elafin. Upon activation by LPS, IRAK associates with TRAF6, and the latter is required for activation of germinal center kinase (GCK), by transiently stabilizing the ubiquitinated GCK polypeptide. GCK then participates in the optimal activation of JNK (27). It may be that elafin somehow perturbs the TRAF6-dependent stabilization of GCK by interfering with the ubiquitin-proteasome machinery that normally regulates GCK. This would account for our observed inhibitory effect of elafin on JNK as opposed to p38 phosphorylation.
Taken together, these observations suggest that elafin’s inhibition of LPS-induced AP-1 and NF-κB activity is due to an inhibitory effect of elafin on the ubiquitin-proteasome pathway. We have previously shown similar effects of SLPI on LPS and LTA-induced IRAK, IκBα and IκBβ degradation (8,9). We have recently demonstrated that SLPI can enter monocytes thus suggesting a direct effect of SLPI on the ubiquitin-proteasome pathway (28). Another antimicrobial peptide, PR-39, has been shown to inhibit TNF-induced NF-κB activation by binding directly to subunits of the proteasome (29). Therefore, it seems likely that the anti-inflammatory activity of SLPI and elafin are due, in part, to their ability to enter cells and act at some point on the ubiquitin-proteasome pathway resulting in delayed turnover of polyubiquinated proteins with repercussions for activation of the NF-κB and AP-1 pathways.

In conclusion, the evidence presented in this study shows that elafin inhibits LPS-induced AP-1 and NF-κB activation through an effect on the ubiquitin-proteasome pathway. Due to its selective expression at mucosal surfaces, as well as in alveolar macrophages, monocytes and neutrophils, elafin’s ability to inhibit LPS signalling may be important in disease states such as cystic fibrosis, pneumonia and acute respiratory distress syndrome. The inhibition of two key inflammatory pathways confirms the importance of elafin as a mediator of the innate immune response.

REFERENCES


FIGURE LEGENDS

Fig. 1. Elafin inhibits LPS-induced MCP-1 production and AP-1 activation in U937 cells. A, Effect of elafin on MCP-1 production. U937s were cultured (1 x 10^6/ml) in medium alone, with LPS (0.1µg/ml), or pre-incubated with elafin (10µg/ml) for 1h at 37 °C followed by incubation with LPS (0.1µg/ml) for 24h. MCP-1 ELISA represents results from three experiments. Elafin down-regulation of LPS-induced AP-1 (B) and NF-κB (C) activation. Nuclear extracts were prepared from U937s cultured in medium alone, with LPS (2µg/ml) or...
pre-incubated with elafin (10µg/ml) followed by activation with LPS (2µg/ml) over a time course shown in minutes. Reaction mixtures containing 5µg of protein and 0.5µL of biotin end-labeled oligonucleotide containing either the AP-1 or NF-κB consensus sequences were resolved by electrophoresis on a 6% polyacrylamide gel. Each EMSA represents results from three experiments. Con, control.

**Fig. 2.** Elafin prevents LPS-induced phosphorylation of ATF2 and c-jun. **A**, Effect of elafin on ATF2 phosphorylation. Nuclear lysates from U937s cultured (2 x 10⁶/ml) in medium alone, with LPS (2µg/ml) or pre-incubated with elafin (10µg/ml) followed by LPS (2µg/ml) over the time courses shown in minutes, were electrophoresed on a 10% SDS-PAGE and probed by Western blot for phosphorylated and total ATF2 antibody, A, and phosphorylated and total c-jun, B. Results shown are representative of three separate experiments. Con, control. Phospho, phosphorylated.

**Fig. 3.** Elafin has no effect on p38 phosphorylation, but inhibits JNK phosphorylation. U937s were cultured (1 x 10⁶/ml) in medium alone, with LPS (2µg/ml) or pre-incubated with elafin (10µg/ml) followed by LPS (2µg/ml) over the time courses indicated, and cytoplasmic, A, and nuclear fractions, B, were subjected to Western analysis for phosphorylated and total p38. Cytoplasmic and nuclear fractions were also analysed for phosphorylated JNK, C, and Lamin B1, D, the latter as a loading control. Results shown are representative of three separate experiments. Con, control. Phospho, phosphorylated.

**Fig. 4.** Elafin prevents LPS-induced degradation of IkBα, IkBβ and IRAK. **A**, U937s were cultured (2 x 10⁶/ml) in medium alone, with LPS (2µg/ml) or pre-incubated with elafin (10µg/ml) followed by activation with LPS (2µg/ml) over the time courses shown in minutes, and cytoplasmic fractions were subjected to Western analysis for IkBα, A, IkBβ, B, and IRAK, C. Results shown represents results of three experiments. Con, control.

**Fig. 5.** Elafin has no effect on LPS-induced phosphorylation of IkBα, or on specified 20 S peptidase activities, but results in increased ubiquitination. U937s were cultured (2 x 10⁶/ml) in medium alone, the remainder were cultured with the proteasome inhibitor ALLN (1µL/ml), either alone and with LPS (2µg/ml) or pre-incubated with elafin (10µg/ml, 1h) followed by LPS (2µg/ml, 30 min). Cytoplasmic lysates were electrophoresed on a 10% SDS-PAGE and probed by Western blot for phosphorylated IkBα, A. Cytoplasmic extracts from cells treated in medium alone, with LPS, or preincubated with elafin were assayed for 20 S peptidase activity, *i.e.* for chymotrypsin-like activity using Suc-LLVY-AMC, peptidylglutamyl peptide hydrolyzing activity using Z-Leu-Leu-Glu-AMC, and for trypsin-like activity using Z-Ala-Arg-Arg-AMC. Extracts were incubated in buffer (25mM HEPES, 5 mM EDTA, 0.1% CHAPS, 5mM ATP, pH 7.5 with 2 mM DTT) for 30 min at 37 °C, and fluorescence was determined by excitation at 355nm and emission at 460nm, B. Cells were also cultured (2 x 10⁶/ml) in medium alone, with LPS (2µg/ml) or pre-incubated with elafin followed by activation with LPS (2µg/ml) over the time courses shown in hours, and cytoplasmic fractions were subjected to Western analysis for ubiquitin, C Results shown are representative of three separate experiments. Con, control. Phospho, phosphorylated, FU, fluorescence units.
Figure 1

A. LPS-induced MCP-1 production +/- elafin

B. AP-1

C. NF-κB
Figure 2

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Phospho ATF2

ATF2

+LPS +Elafin+LPS

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Phospho c-jun

C-jun

+LPS +Elafin+LPS
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Phospho p38

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Phospho p38

p38

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Phospho JNK

JNK

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Phospho JNK

Lamin B1

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Figure 4

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+LPS

+Elafin + LPS

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+LPS

+Elafin + LPS

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+LPS

+Elafin + LPS

Figure 5.
A. 

+ALLN

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Phospho IkBα

B. 

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+LPS

Effect of elafin on LPS induced LLE-AMC turnover

C. 

Effect of elafin on LPS induced ARR-AMC turnover

Effect of elafin on LPS induced LLVY-AMC turnover

\[
\text{Effect of elafin on LPS induced ARR-AMC turnover} \quad \text{Effect of elafin on LPS induced LLE-AMC turnover}
\]