Elafin Prevents Lipopolysaccharide-induced AP-1 and NF-κB Activation via an Effect on the Ubiquitin-Proteasome Pathway*

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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-induced neutrophil influx into murine alveoli as well as to abrogate lipopolysaccharide-induced production of matrix metalloprotease 9, macrophage inhibitory protein 2, and tumor necrosis factor-α by as-yet unidentified mechanisms. In this report we have shown that elafin inhibits the lipopolysaccharide-induced production of monocyte chemoattractant protein-1 in monocytes by inhibiting AP-1 and NF-κB activation. Elafin prevented lipopolysaccharide-induced phosphorylation of AP-1, c-Jun, and JNK but had no effect on phosphorylation of p38. The lipopolysaccharide-induced degradation of IL-1R-associated kinase 1, IκBα, and IκBβ was inhibited by elafin but phosphorylation of IκBα was unaffected. Polyubiquitinated protein including polyubiquitinated IκBα was shown to accumulate in the presence of elafin. These results suggest that inhibition by elafin of lipopolysaccharide-induced AP-1 and NF-κB activation occurs via an effect on the ubiquitin-proteasome pathway.

Elafin is a 6-kDa serine anti-protease initially identified in psoriatic skin scales and is also called skin-derived anti-leucoprotease. Subsequently, “elastase-specific inhibitor” was isolated from human sputum and found to have identical N-terminal sequencing as skin-derived anti-leucoprotease, and the term elafin was suggested (1). Elafin is currently known to be expressed in airways, other mucosal surfaces such as esophagus, vagina, endometrium, and 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 characterized by possessing a C-terminal core domain consisting of four disulfide bonds. Also known as trappins (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, proteinase 3, and porcine pancreatic elastase and is induced by cytokine and other stimuli including interleukin 1β, tumor necrosis factor, human neutrophil elastase, and lipopolysaccharide (LPS) (4). 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 κ B (NF-κB) (3), whereas in mammary epithelial cells the transcription factor activating protein-1 (AP-1) mediates transcriptional activation (4). 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 that is independent of its anti-elastase activity or charge properties (5).

In light of these results, we have looked at the modulatory effects of elafin on LPS signaling in U937 cells, including the effect of elafin on LPS-induced monocyte chemoattractant protein-1 (MCP-1). MCP-1 is a chemokine produced by mononuclear phagocytes and is a potent activator of monocyte function.

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2 The abbreviations used are: LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; SLPI, secretory leucocyte protease inhibitor; ALLN, N-acetyl-Leu-Leu-norleucinal; AMMC, 7-amino-4-methylcoumarin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; IRAK, IL-1R-associated kinase; JNK, c-Jun N-terminal kinase; Z, benzoyloxycarbonyl.
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**EXPERIMENTAL PROCEDURES**

**Materials**—RPMI 1640 medium was obtained from Invitrogen, 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. Antibody to ubiquitin was purchased from Sigma. Western blotting reagents were obtained from Pierce. Fluorogenic substrates for 20 S proteasome activity assays were purchased from Merck Biosciences.

**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 × 10⁶ cells/ml), the cells were washed in ice-cold phosphate-buffered saline and resuspended in a hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM dithiothreitol). Cells were pelleted by centrifugation at 14,000 g for 10 min at 4 °C and then lysed for 10 min on ice in 20 μl of hypotonic buffer containing 0.1% Igepal CA-630, 1 mM sodium vanadate, 50 mM β-glycerophosphate, and 1× complete protease inhibitor mixture (Roche Applied Science). Lysates were centrifuged as before, and the supernatant (cytoplasmic fraction) was retained for Western analysis (see below). The remaining nuclear pellet was lysed in 15 μl of lysis buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 50 mM β-glycerophosphate, and 1× 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). 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 dithiothreitol, 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).

**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 each for phosphorylated ATF2 and phosphorylated c-Jun and 40 μg each for 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 phosphate-buffered saline plus 0.05% Tween-20 (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 horseradish peroxidase-conjugated secondary antibodies (1:2000). Antibody-antibody complexes were detected with enhanced chemiluminescence reagents (Pierce).

**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 dipeptidylaminopeptidase IV hydrolyzing activity), and Z-Ala-Arg-Arg-AMC (for trypsin-like activity). Treated cells were lysed in 700 μl of 25 mM HEPES, 5 mM EDTA, 0.1% CHAPS, 5 mM ATP, pH 7.5, with 2 mM dithiothreitol (21). Each sample was incubated with each substrate (50 μM, final concentration) in lysis buffer for 30 min at 37 °C, and fluorescence (substrate turnover) was determined by excitation at 355 nm and emission at 460 nm.

FIGURE 1. Elafin inhibits LPS-induced MCP-1 production. U937s were cultured (1 × 10⁶/ml) in medium alone, with LPS (0.1 μg/ml), or preincubated with elafin (10 μg/ml) for 1 h at 37 °C for 24 h. Enzyme-linked immunosorbent assay represents results from three experiments.

(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 the effect of elafin upon the ubiquitin-proteasome pathway.
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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 (10 μg/ml) for 1 h followed by incubation with LPS. LPS was found to induce significantly more MCP-1 production in U937 cells compared with cells incubated in medium alone. Elafin inhibited LPS-induced MCP-1 production (Fig. 1). 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 preincubating U937 cells with elafin (1 h) followed by LPS over the time courses shown (Fig. 2, A and B). LPS was shown to induce significantly more AP-1 activation in U937 cells (Fig. 2A, left, lanes 30, 60, and 120) compared with cells incubated in medium alone (Fig. 2A, left, lane Con). Elafin prevented LPS-induced AP-1 activation across the time course (Fig. 2A, right). Similarly, LPS induced significantly more NF-κB activation (Fig. 2B, left, lanes 15, 30, and 60) versus control (Fig. 2B, lane Con), which was markedly inhibited by elafin (Fig. 2B, 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. 2C, top panel, left), and this phosphorylation was significantly abrogated by preincubation with elafin (Fig. 2C, upper panel, right). Protein loading was controlled for by probing the stripped blot for total ATF2 antibody (C, top panel, lower panel, left), and nuclear fractions were analyzed for phosphorylated JNK and lamin B1 (D, the latter as a loading control). Results shown are representative of three separate experiments. Con, control. Phospho, phosphorylated.

FIGURE 2. Elafin down-regulation of LPS-induced AP-1 (A) and NF-κB (B) activation. Nuclear extracts were prepared from U937s cultured in medium alone, with LPS (2 μg/ml), or preincubated with elafin (10 μg/ml) followed by activation with LPS (2 μg/ml) over a time course shown in minutes. Reaction mixtures containing 5 μg of protein and 0.5 μl 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 electrophoretic mobility shift assay represents results from three experiments. Con, control. C and D, elafin prevents LPS-induced phosphorylation of ATF2 and c-Jun. Nuclear lysates from U937s cultured (2 × 10⁶/ml) in medium alone, with LPS (2 μg/ml), or preincubated with elafin (10 μg/ml) followed by LPS (2 μg/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 (C) and phosphorylated and total c-Jun (D). Results shown are representative of three separate experiments. Con, control. Phospho, phosphorylated.

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

FIGURE 4. Elafin prevents LPS-induced degradation of IκBα, IκBβ, and IRAK. A, U937s were cultured (2 × 10⁶/ml) in medium alone, with LPS (2 μg/ml), or preincubated with elafin (10 μg/ml) followed by activation with LPS (2 μg/ml) over the time courses shown in minutes. Cytoplasmic fractions were subjected to Western analysis for IκBα (A), IκBβ (B), and IRAK (C). Results shown represent results of three experiments. Con, control.
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Elafin has no effect on LPS-induced phosphorylation of IkBα or on specified 20 S peptidase activities but results in increased polyubiquitination. A, U937s were cultured (2 × 10^6/ml) in medium alone or with the proteasome inhibitor ALLN (1 μ/ml) either alone, with LPS (2 μg/ml), or preincubated with elafin (10 μg/ml, 1 h) followed by LPS (2 μg/ml, 30 min). Cytoplasmic lysates of duplicate samples were electrophoresed on a 10% SDS-PAGE and probed by Western blot for phosphorylated IkBα. Lanes 1 and 2, control lysates; lanes 3 and 4, control + ALLN lysates; lanes 5 and 6, LPS + ALLN lysates; lanes 7 and 8, Elafin/LPS + ALLN lysates. B, 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-LLE-AMC, and for trypsin-like activity using Z-ARR-AMC. Extracts were incubated in buffer (25 mM HEPES, 5 mM EDTA, 0.1% CHAPS, 5 mM ATP, pH 7.5 with 2 mM dithiothreitol) for 30 min at 37 °C, and fluorescence was determined by excitation at 355 nm and emission at 460 nm. C, cells were also cultured (2 × 10^6/ml) in medium alone, with LPS (2 μg/ml), elafin alone, or preincubated with elafin followed by activation with LPS (2 μg/ml) for 4 h, and cytoplasmic fractions were subjected to Western analysis for ubiquitin and IkBα. Results shown are representative of three separate experiments. Con, control. Phospho, phosphorylated. FU, fluorescence unit.

To investigate whether elafin affects the phosphorylation of IkBα, cells were treated with or without the proteasome inhibitor ALLN (100 μg/ml for 30 min), used to stabilize the labile phosphorylated IkBα. 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 IkBα was observed in all samples treated with ALLN (Fig. 5A, lanes 3–8), although only weakly so in the samples not also treated with LPS (Fig. 5A, lanes 3–4). The increased phosphorylation of IkBα 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, IkBα, and IkBβ undergo ubiquitination in response to stimuli such as LPS and because elafin did not appear to prevent the LPS-induced phosphorylation of these proteins (Fig. 4, A–C, right rows), we assessed the effect of elafin on NF-κB regulatory proteins, degradation of IL-1R-associated kinase (IRAK), IkBα, and IkBβ were assessed. U937 cells were again either incubated with medium alone, with LPS, or preincubated with elafin followed by LPS over the time courses shown (Fig. 4). Western blot analyses of cytoplasmic extracts showed LPS-induced degradation of IkBα, IkBβm 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).
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phosphorylation of IkBα, we assessed the possible effect of elafin on proteasome function. First, the effect of elafin on peptidase activity associated with the 20 S proteasome was determined. Elafin was found not to affect any of the peptidase activities assessed (Fig. 5B). However, LPS-induced polyubiquitination appeared to increase in the presence of elafin/LPS compared with when stimulated with LPS or elafin alone (Fig. 5C) as assessed by Western blot analysis, indicating an effect of elafin on turnover of polyubiquitinated proteins. Finally, polyubiquitinated IkBα was also found to accumulate in the presence of elafin/LPS compared with LPS and elafin alone, indicating a specific effect of elafin on the turnover of IkBα following LPS stimulation (Fig. 5C).

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 tumor necrosis factor receptor-associated factor (TRAF)-6 resulting in activation of the mitogen-activated protein 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 κB (IkB) 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 prevented the LPS-induced degradation of the NF-κB regulatory proteins IRAK, IkBα, and IkBβ. These proteins are regulated by phosphorylation, ubiquitination, and proteasomal degradation (25). Although elafin appeared to have no effect on LPS-induced phosphorylation of IkBα, we found that elafin appears to delay ubiquitination of proteins in response to LPS, which corresponded to the inhibition of LPS-induced IRAK, IkBα, and IkBβ degradation in the presence of elafin. Upon activation by LPS, IRAK associates with TRAF6, and the latter is required for activation of germlinal center kinase, by transiently stabilizing the ubiquitinated germlinal center kinase polypeptide. Germlinal center kinase then participates in the optimal activation of JNK (27). It may be that elafin somehow perturbs the TRAF6-dependent stabilization of germlinal center kinase by interfering with the ubiquitin-proteasome machinery that normally regulates germlinal center kinase. This would account for our observed inhibitory effect of elafin on JNK as opposed to p38 phosphorylation.

Taken together, these observations suggest that inhibition by elafin 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 lipoteichoic acid-induced IRAK, IkBα, and IkBβ 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 affecting proteasome function (29). Therefore, it seems likely that the anti-inflammatory activity of SLPI and elafin is due, in part, to their ability to enter cells and act at some point on the ubiquitin-proteasome pathway, resulting in delayed turnover of polyubiquitinated proteins with repercussions for activation of the NF-κB and AP-1 pathways. However, peptidase activities associated with the 20 S proteasome were not affected by elafin.

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, the ability of elafin to inhibit LPS signaling 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.

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