13-9-2002

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Citation

Taggart CC, Greene CM, McElvaney NG, O'Neill S. Secretory leucoprotease inhibitor prevents lipopolysaccharide-induced IkappaBalpha degradation without affecting phosphorylation or ubiquitination. Journal of Biological Chemistry. 2002;277(37):33648-53.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 37, Issue of September 13, pp. 33648–33653, 2002

Printed in U.S.A.

Secretory Leucoprotease Inhibitor Prevents Lipopolysaccharide-induced IκBα Degradation without Affecting Phosphorylation or Ubiquitination*

Received for publication, April 17, 2002, and in revised form, June 19, 2002
Published, JBC Papers in Press, June 25, 2002, DOI 10.1074/jbc.M203710200

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Secretory leucoprotease inhibitor (SLPI) is a non-glycosylated protein produced by epithelial cells, macrophages, and neutrophils and was initially identified as a serine protease inhibitor of the neutrophil proteases elastase and cathepsin G. In addition to its antiprotease activity, SLPI has been shown to exhibit anti-inflammatory properties including down-regulation of tumor necrosis factor-α expression by lipopolysaccharide (LPS) in monocytes, inhibition of NF-κB activation by IgG immune complexes in a rat model of acute lung injury, and prevention of human immunodeficiency virus infectivity in monocytic cells via as yet unidentified mechanisms. In this report we have shown that SLPI prevents LPS-induced NF-κB activation by inhibiting degradation of IκBα without affecting the LPS-induced phosphorylation and ubiquitination of IκBα. We have also demonstrated that SLPI prevents LPS-induced interleukin-1 receptor-associated kinase and IκBβ degradation. In addition, we have demonstrated that oxidized SLPI, a variant of SLPI that has diminished antiprotease activity, cannot prevent LPS-induced NF-κB activation or Inhibitor κB α/β degradation indicating that the anti-inflammatory effect of SLPI on the LPS-signaling pathway is dependent on its antiprotease activity. These results suggest that SLPI may be inhibiting proteasomal degradation of NF-κB regulatory proteins, an effect that is dependent on the antiprotease activity of SLPI.

Secretory leucoprotease inhibitor (SLPI)[1] is an 11.7-kDa non-glycosylated protein, which is expressed by epithelial cells, macrophages, and neutrophils (1–3). It is found in various secretory fluids but primarily bronchial and nasal secretions (4, 5). SLPI forms inhibitory complexes with a variety of proteolytic enzymes including neutrophil elastase and cathepsin G and therefore appears to be an important component of the antiprotease defense of the lung (6). The amino acid sequence of SLPI and the resulting NMR solution structure have revealed a protein composed of two highly homologous domains of 53 and 54 amino acids, 8 disulfide bridges in total, and a large number of positively charged residues (7). The small size of SLPI and the large number of disulfide bridges were thought to confer resistance of SLPI to proteolysis. However, we have demonstrated recently (8) that SLPI is susceptible to proteolytic cleavage by members of the elastolytic cathepsin family and that cathepsin L present in the emphysematous lung results in SLPI cleavage and inactivation of its antiprotease activity.

Recently, it has been demonstrated that SLPI also possesses anti-inflammatory, anti-viral, and anti-bacterial activity. LPS-hyporesponsive cells have been shown to transcribe SLPI, and transfection of macrophages with SLPI was shown to suppress LPS-induced activation of NF-κB and production of nitric oxide and TNFα by an unknown mechanism (9). In addition, IFNγ suppressed expression of SLPI and restored LPS responsiveness to SLPI-producing cells (9). SLPI has also been shown to inhibit HIV infectivity of monocytes by blocking viral DNA synthesis by a mechanism that does not involve binding to HIV directly but is most likely due to interaction with the host cell (10, 11).

In a model of acute lung injury, induced by intrapulmonary deposition of IgG immune complexes in rats, prior administration of SLPI attenuated pulmonary recruitment of neutrophils and decreased lung injury (12). In addition, prior administration of SLPI to these animals resulted in greatly reduced NF-κB activation in whole lung samples, although interestingly down-regulation of NF-κB activation was not observed in alveolar macrophages isolated by bronchoalveolar lavage from these animals (12). Further investigation of the NF-κB regulatory proteins revealed that IκBβ degradation was prevented in animals pretreated with SLPI. These data suggest that the inhibitory effects of SLPI are selective for the signal transduction pathway leading to NF-κB activation.

With these results as background, we have further investigated the effects of SLPI inhibition on LPS-induced NF-κB activation in U937 cells. In this report we show that SLPI inhibits LPS-induced NF-κB activation in U937 cells by preventing degradation of IκBα but without affecting the phosphorylation or ubiquitination of IκBα. In the presence of SLPI, phosphorylated and ubiquitinated IκBα appears to accumulate. We have also demonstrated the prevention of degradation of IRAK and IκBβ by LPS in U937 cells. Finally, we have shown that SLPI does not affect 20 S proteasome peptidase-related activity. Given that IRAK, IκBα, and IκBβ are phosphorylated and degraded by the proteasome following activa-

* This work was supported by the Health Research Board of Ireland, the Higher Education Authority of Ireland, the Charitable Infirmary Charitable Trust, and the Royal College of Surgeons in Ireland. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: SLPI, secretory leucoprotease inhibitor; IRAK, interleukin-1 receptor-associated kinase; NF-κB, nuclear factor κB; LPS, lipopolysaccharide; ALLN, *N*-acetyl-Leu-Leu-norleucinal; AMC, 7-amino-4-methylcoumarin; TNF, tumor necrosis factor; HIV, human immunodeficiency virus; Z, benzyloxycarbonyl; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; Suc, succinyl.

tion with LPS, these results would suggest that SLPI is inhibiting the ubiquitin-proteasome pathway either directly or indirectly.

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 SLPI was obtained from R & D Systems (Abingdon, Oxon, UK). Antibodies to IRAK and IκBβ were obtained from BD Transduction Laboratories (Heidelberg, Germany) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Antibodies to IκBα (native and phosphorylated forms) were from New England Biolabs (Hitchin, Herts, UK). Anti-ubiquitin antibody was purchased from Affiniti Research Products Ltd. (Mamhead, Exeter, UK). Western blotting reagents were obtained from Tropix (Bedford, MA). Z-ARR-AMC, Z-LLE-AMC, Suc-LLVY-AMC, and the proteasome inhibitors, ALLN and MG-132, were from CN Biosciences (Beeston, Nottingham, UK). All other general reagents were from Sigma.

EMSA—Nuclear extracts (5 μg) were incubated with 10,000 cpm of [γ-^{32}P]ATP (Amersham Biosciences) T4 polynucleotide kinase (Promega, Madison, WI) end-labeled oligonucleotide containing the NF-κB consensus sequence (Santa Cruz Biotechnology). 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). In some experiments, unlabeled wild-type or mutant NF-κB oligonucleotide (Santa Cruz Biotechnology) was added to extracts before incubation with the labeled oligonucleotide. Reaction mixtures were electrophoresed on native 5% polyacrylamide gels that were subsequently dried and autoradiographed.

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% CO_2.

Preparation of Oxidized SLPI—One hundred micrograms of recombinant SLPI were incubated for 2 h at room temperature in 500 μl of reaction mixture containing 50 mM potassium phosphate, 100 mM potassium chloride, 1 mM magnesium chloride, pH 5.0, and 20 mM hydrogen peroxide (6%; Ovelle, Dundalk, Ireland). At this pH methionine is oxidized selectively to methionine sulfoxide (13). At the end of the reaction, the hydrogen peroxide was removed by desalting the mixture, twice, through a Microcon-3 column (Millipore Corp., Bedford, MA). Protein concentration of the sample was measured by Bradford assay before and after desalting to ensure no loss of SLPI during the desalting process. The antiprotease activity of the sample was determined by titrating increasing amounts of oxidized SLPI against a fixed standard of neutrophil elastase of known activity. Residual neutrophil elastase activity was measured using the substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (14).

Preparation of Nuclear Extracts—After treating cells with the indicated reagents for the indicated times in 24-well plates (1×10^6 cells/ml), the cells were washed with ice-cold phosphate-buffered saline and resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 1× complete protease inhibitor mixture (Roche Molecular Biochemicals)). Cells were pelleted by centrifugation at 13,000 × *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. 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 $MgCl_2$, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride) for 15 min on ice. After centrifugation at 13,000 × *g* for 15 min at 4 °C, nuclear extracts were removed into 50 μl of storage buffer (10 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 20% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT). Protein concentrations were determined (15), and the extracts were stored at −80 °C.

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 (15). Equal amounts of protein from each sample (10 μg for each of IRAK, IκBα, and IκBβ) were electrophoresed by SDS-PAGE and blotted. Transferred proteins were blocked in I-Block (Tropix, Bedford, MA) in PBST. Proteins were detected using primary antibodies directed against IRAK (1:500), IκBα (1:1000), phosphorylated IκBα (1:2000), and IκBβ (1:1000) followed by incubation with alkaline phosphatase-conjugated secondary antibodies (1:7500). Antigen-antibody complexes were detected with enhanced chemiluminescence reagents (Tropix, Bedford, MA). Phosphorylated IκBα levels were quantified by densitometry using GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, UK) and Syngene GeneSnap and GeneTools software.

Immunoprecipitation of Phosphorylated IκBα—Cytoplasmic extracts (200 μg of protein), made up to 100 μl with buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM sodium vanadate, and 1× complete protease inhibitor mixture (Roche Molecular Biochemicals)), were incubated overnight at 4 °C with anti-phosphorylated-IκBα IgG (1:100). The samples were then treated with 30 μl of protein A-agarose beads for 2 h at 4 °C after which the samples were centrifuged and washed 5 times in buffer. The beads were boiled in SDS-PAGE sample treatment buffer and electrophoresed on a 10% SDS-PAGE. The gel was blotted and incubated with a monoclonal antibody to ubiquitin (1:1000 for 1 h) followed by incubation with an alkaline phosphatase-labeled secondary antibody (1:7500). Antigen-antibody complexes were detected with enhanced chemiluminescence reagents (Tropix, Bedford, MA).

20 S Proteasome Activity Assays—The various 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 hydrolyzing activity), and Z-Ala-Arg-Arg-AMC (for trypsin-like activity). Treated cells were lysed in 250 μl of 25 mM HEPES, 5 mM EDTA, 0.1% CHAPS, 5 mM ATP, pH 7.5, with 2 mM DTT (16). Equal amounts of protein from each sample were 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. In some experiments, a further aliquot of SLPI (10 μg/ml) was added to the cell extract of cells already incubated with SLPI/LPS, and 20 S peptidase activity was measured as above.

RESULTS

SLPI Abrogation of LPS-induced NF-κB Activation—U937 cells were incubated with LPS (0.1 μg/ml) for 2 h following time course and dose-response experiments (data not shown). Some samples were preincubated with SLPI at various concentrations (0.1, 1.0, and 10 μg/ml) for 1 h followed by incubation with LPS (0.1 μg/ml). LPS was found to induce significantly more NF-κB nuclear localization in U937 cells (Fig. 1*a*, *lane 2*) compared with cells incubated in medium alone (Fig. 1*a*, *lane 1*). SLPI inhibited LPS-induced NF-κB activation in a dose-dependent manner (Fig. 1*a*, *lanes 3–5*). These experiments were repeated three times with similar results.

U937 cells were also preincubated with oxidized SLPI (10 μg/ml) followed by incubation with LPS (0.1 μg/ml) for 2 h. Oxidized SLPI (10 μg/ml) did not prevent LPS-induced NF-κB activation (Fig. 1*b*, *lanes 6* and *7 versus lanes 2* and *3*, LPS alone) compared with cells preincubated with native SLPI (10 μg/ml) (Fig. 1*b*, *lanes 4* and *5*).

Effect of SLPI on LPS-induced Degradation of IRAK, IκBα, and IκBβ—U937s were incubated in medium alone or with LPS (1.0 μg/ml) alone or following preincubation with SLPI (10 μg/ml, 1 h) or oxidized SLPI (10 μg/ml, 1 h) for 30, 60, 120, and 180 min. Western blot analysis of cytoplasmic extracts showed LPS-induced degradation of IRAK, IκBα, and IκBβ by 120 min (Fig. 2, *a–c, top rows*), whereas preincubation of U937s with native SLPI resulted in inhibition of the degradation of IRAK, IκBα, and IκBβ at 120 min (Fig. 2, *a–c*, *middle rows*). Preincubation of U937s with oxidized SLPI followed by activation with LPS gave a similar pattern of IRAK, IκBα, and IκBβ degradation by 120 min when compared with LPS alone (Fig. 2, *a–c*, *bottom rows versus* Fig. 2, *a–c*, *top rows*). These results demonstrate that SLPI prevents LPS-induced degradation of IRAK, IκBα, and IκBβ, but oxidized SLPI has lost the ability to prevent LPS-induced degradation.

LPS-induced Phosphorylation and Ubiquitination of IκBα—To assess the effect of SLPI on LPS-induced phosphorylation of NF-κB regulatory proteins, phosphorylation of IκBα was assessed. U937s were initially incubated with the protea-

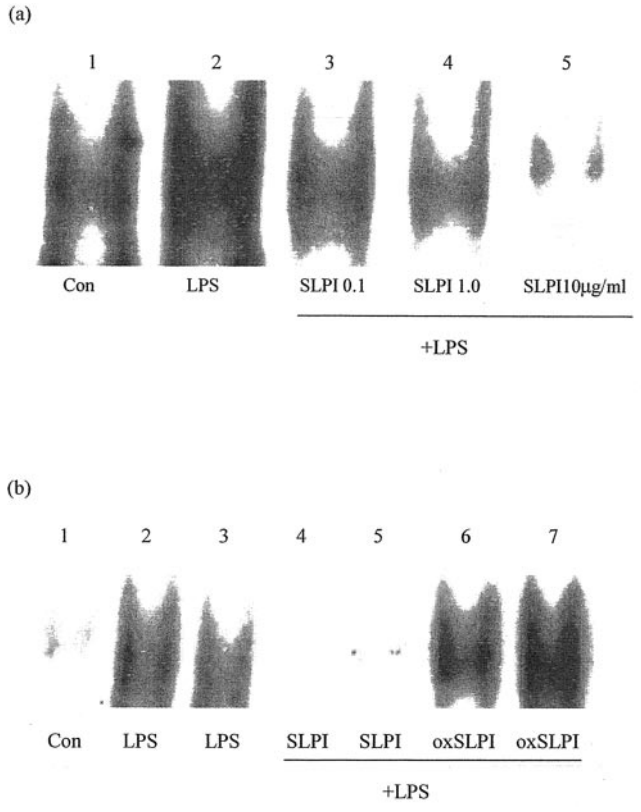

FIG. 1. *a*, SLPI down-regulation of LPS-induced NF-κB activation in U937 cells. U937s were cultured (1×10^6/ml) in medium alone, with LPS (0.1 μg/ml), or preincubated with varying concentrations of SLPI (0.1, 1.0, and 10 μg/ml) for 1 h at 37 °C followed by incubation with LPS (0.1 μg/ml) for 2 h. Nuclear extracts were prepared, and reaction mixtures containing 5 μg of protein and 10,000 cpm [γ-^{32}P]ATP end-labeled NF-κB consensus sequence were resolved by electrophoresis on a 5% polyacrylamide gel. EMSA represents results from three experiments. *b,* U937s were cultured (1×10^6/ml) in medium alone, with LPS (0.1 μg/ml), or preincubated with oxSLPI or wild-type SLPI (10 μg/ml) for 1 h at 37 °C followed by incubation with LPS (0.1 μg/ml) for 2 h. Nuclear extracts were prepared, and NF-κB DNA-protein complexes were resolved on a 5% polyacrylamide gel. EMSA represents results from three experiments. *Con*, control.

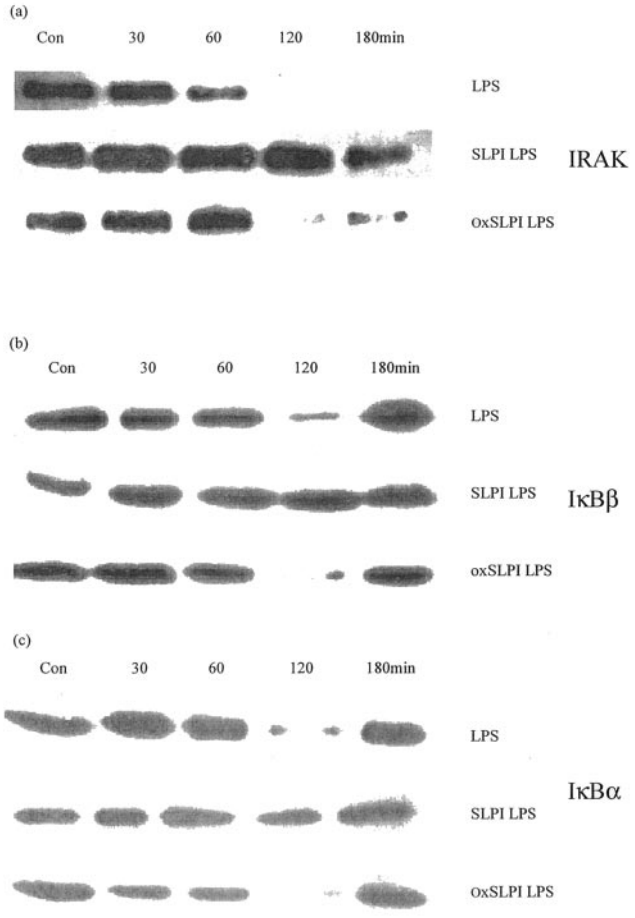

FIG. 2. **SLPI prevents LPS-induced degradation of IRAK, IκBα, and IκBβ.** *a*, effect of SLPI on IRAK degradation. 10 μg of lysates prepared from cells incubated in medium alone, with LPS, or preincubated with SLPI (10 μg/ml) or oxSLPI (10 μg/ml) followed by activation with LPS (1 μg/ml) over a time course of 0, 30, 60, 120, and 180 min were assayed for IRAK. The same samples were assayed for IκBα (*b*) and IκBβ (*c*). Each experiment was repeated four times with similar results. *Con*, control.

some inhibitor ALLN (100 μg/ml) for 30 min to stabilize the labile phosphorylated IκBα. Cells were incubated in medium alone, preincubated with SLPI or oxidized SLPI (10 μg/ml) alone, or stimulated with LPS (1 μg/ml) for 30 min. Phosphorylation of IκBα was observed in all samples treated with LPS (Fig. 3*a*, *lanes 3–5*) but not in the control sample (Fig. 3*a*, *lane 1*, the only sample not preincubated with ALLN). Some phosphorylated IκBα was detected in the control sample treated with ALLN (Fig. 3*a*, *lane 2*) and the samples incubated with SLPI or oxidized SLPI alone (Fig. 3*a*, *lanes 6* and *7*). Densitometric analysis of three experiments revealed that incubation of cells with SLPI/LPS (+ALLN) resulted in a 9.56 ± 0.04-fold increase in the levels of phosphorylated IκBα compared with control (+ALLN) (Fig. 3*b*). This is compared with an increase of 3.68 ± 0.31- and 3.74 ± 0.54-fold for LPS (+ALLN) and oxidized SLPI/LPS (+ALLN), respectively, compared with control (+ ALLN) (Fig. 3*b*). Cells treated with SLPI and oxidized SLPI alone (+ALLN) did not result in an increase in phosphorylated IκBα above control (+ALLN) (Fig. 3*b*). Therefore, incubation of cells with SLPI, in the presence of LPS, resulted in increased amounts of phosphorylated IκBα compared with cells treated with LPS or oxidized SLPI/LPS.

Effect of SLPI on Ubiquitination—To investigate whether accumulation of phosphorylated IκBα in the presence of SLPI also results in accumulation of ubiquitinated, phosphorylated IκBα, cells were treated with ALLN followed by preincubation with SLPI/oxidized SLPI and subsequent incubation with LPS. Equal amounts of cell extract were electrophoresed, blotted, and incubated with anti-phosphorylated IκBα. The blot was overdeveloped to ensure visualization of ubiquitinated IκBα. High molecular weight bands (~200 kDa) were observed in the samples treated with SLPI (Fig. 3*c*, *lanes 4* and *5*) that were not present in the other lanes. To confirm that the high molecular weight bands in the SLPI-treated samples were ubiquitinated forms of phosphorylated IκBα, we immunoprecipitated phosphorylated IκBα from samples treated with SLPI, oxidized SLPI, or LPS in the presence of ALLN. The resulting blot was incubated with anti-ubiquitin and revealed the presence of a high molecular weight band in the SLPI-treated lane (Fig. 3*d*, *lane 3*) of the same size as that present in phosphorylated IκBα blot (Fig. 3*c*, *lanes 4* and *5*). The same high molecular weight band was also present in the LPS and oxidized SLPI/LPS-treated lanes (Fig. 3*d*, *lane 2* and *4*) although to a much lesser degree. This confirmed that the high molecular weight band is a polyubiquitinated form of phosphorylated IκBα.

Effect of SLPI on 20 S Peptidase Activity—To investigate further the possible effect of SLPI on various peptidase activities associated with the 20 S proteasome, cells were lysed and peptidase activity measured. Chymotrypsin-like activity (Suc-LLVY-AMC), peptidylglutamyl peptide hydrolyzing activity

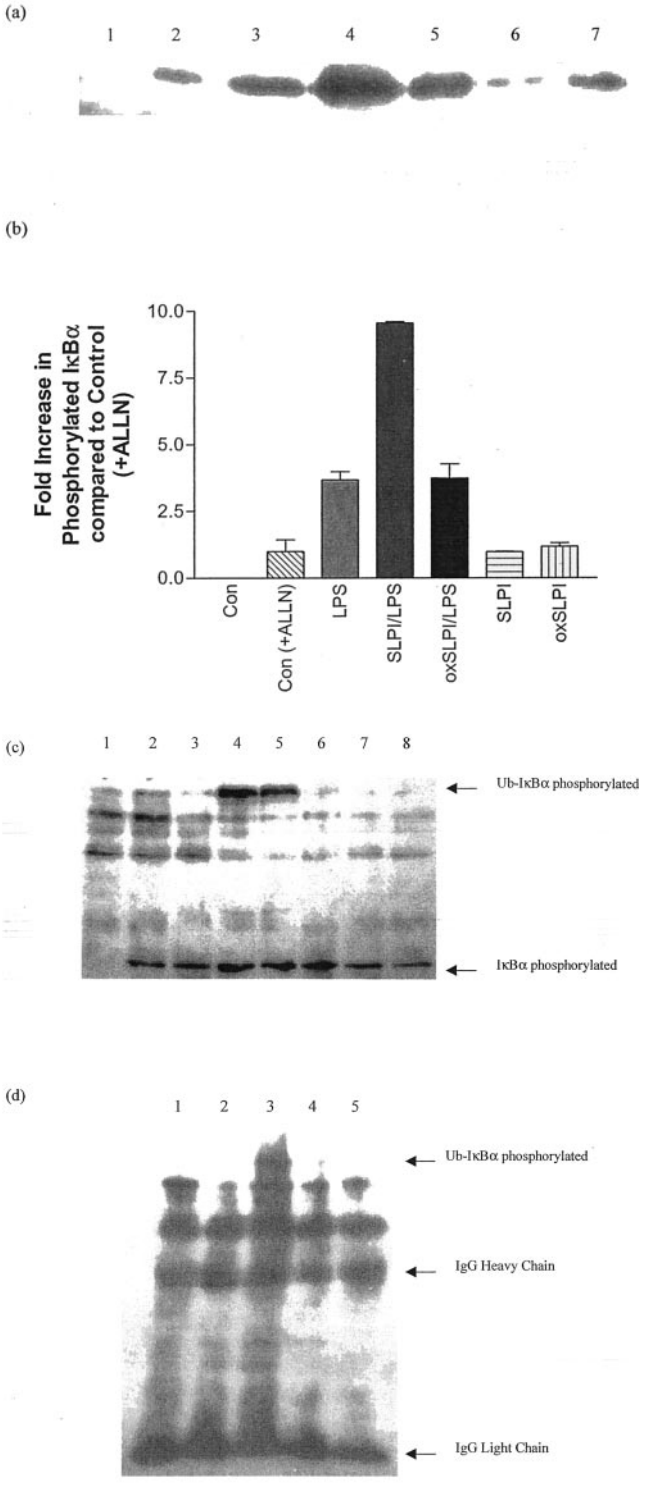

FIG. 3. **SLPI does not inhibit IκBα phosphorylation and ubiquitination.** *a*, U937s (1×10^6/ml) were incubated with ALLN (100 μg/ml) for 30 min followed by incubation in medium alone, with LPS (1 μg/ml for 30 min), SLPI alone (10 μg/ml), oxidized SLPI alone (10 μg/ml), or incubation with SLPI or oxSLPI for 1 h followed by LPS for 30 min. Cytoplasmic extracts were electrophoresed on a 10% SDS-PAGE and probed for phosphorylated IκBα. Each experiment was repeated three times. *Lane 1*, control; *lane 2*, control + ALLN; *lane 3*, LPS 30 min; *lane 4*, SLPI/LPS 30 min; *lane 5*, oxSLPI/LPS 30 min; *lane 6*, SLPI; *lane 7*, oxidized SLPI. Samples in *lanes 2–7* were preincubated with ALLN. *b*, densitometric analysis of *a*. Results from three separate experiments were analyzed by using Syngene GeneSnap and GeneTools software and presented as fold increase in phosphorylated IκBα compared with control (*Con+ALLN*) which was given a value of 1.0. *c*, U937s (1×10^6/ml) were incubated with ALLN (100 μg/ml) for 30 min followed by incubation in medium alone, with LPS (1 μg/ml for 30 min), or incubation with SLPI or oxSLPI for 1 h followed by LPS for 30 min. Cytoplasmic extracts were electrophoresed and assayed for phosphorylated IκBα. On this occasion the blot was overexposed to reveal the presence of a high molecular weight band in *lanes 4* and *5* presumed to be polyubiquitinated, phosphorylated IκBα. Each experiment was repeated three times. *Lane 1*, control; *lanes 2* and *3*, LPS; *lanes 4* and *5*, SLPI/LPS; *lanes 6* and *7*, oxSLPI/LPS; *lane 8*, control. Samples in *lanes 2–8* were preincubated with ALLN. *d*, phosphorylated IκBα was immunoprecipitated from cytoplasmic samples treated as in *c*. Samples were incubated with anti-phosphorylated IκBα IgG overnight at 4 °C followed by incubation with protein A-agarose for 2 h at 4 °C. Ubiquitinated, phosphorylated IκBα is shown in *lanes 2* and *3* and corresponds to the high molecular weight bands seen in *lanes 4* and *5* of *b*. Each experiment was repeated three times. *Lane 1*, control; *lane 2*, LPS; *lane 3*, SLPI/LPS; *lane 4*, oxSLPI/LPS; *lane 5*, control. Samples in *lanes 2–5* were preincubated with ALLN.

(Z-LLE-AMC), and trypsin-like activity (Z-ARR-AMC) were not significantly decreased in those samples treated with SLPI compared with control and LPS-treated samples (Fig. 4). Samples treated with the proteasome inhibitor, MG-132, exhibited significantly decreased activity for all three 20 S peptidases examined (Fig. 4). To confirm that any potential inhibitory effect of SLPI on 20 S peptidase activity was not lost during cell extract preparation, cells that had been treated with SLPI/LPS, followed by cell extract preparation, were further incubated with SLPI (10 μg/ml) and 20 S activities measured. As can be seen from Fig. 4 (SLPI + SLPI/LPS), re-incubation with SLPI did not decrease any of the proteasomal peptidase activities measured confirming that SLPI did not inhibit 20 S activity of the proteasome.

DISCUSSION

SLPI inhibits LPS-induced NF-κB activation in the U937 monocytic cell line by preventing degradation of the regulatory proteins, IRAK, IκBα, and IκBβ. The antiprotease activity of SLPI appears to be required for this mechanism of inhibition because oxidized SLPI (which does not possess antiprotease activity) cannot prevent LPS-induced NF-κB activation nor prevent IRAK, IκBα, and IκBβ degradation. In addition, SLPI does not inhibit LPS-induced phosphorylation or ubiquitination of IκBα but does, in fact, lead to an accumulation of phosphorylated, ubiquitinated IκBα. Finally, SLPI does not inhibit any of the 20 S-associated peptidase activities of the proteasome. Therefore, it would appear that SLPI is exerting its affect by inhibiting, directly or indirectly, the ubiquitin-proteasome pathway by a mechanism dependent on its antiprotease activity.

SLPI has been shown previously to act as an anti-inflammatory mediator by inhibiting HIV infection of monocytic cells (10) and LPS-induced TNFα and MMP-9 expression by monocytes (9, 17). In addition, SLPI has been demonstrated to prevent NF-κB activation in the lungs of rats challenged with IgG immune complexes (12). In an extension to this study, analogues of SLPI, mutated at the active site residue, were shown to have varying effects on IgG immune complex-induced NF-κB activation (18). A SLPI mutant possessing a lysine at position 72 was more active than the wild-type molecule at suppressing intrapulmonary activation of NF-κB (18).

SLPI has also been shown to up-regulate the production of certain anti-inflammatory proteins including hepatocyte growth factor- and LPS-induced interleukin-10 (19, 20). In the former study, it was demonstrated that unlike the wild-type SLPI protein, oxidized SLPI did not up-regulate hepatocyte growth factor (19). In this report we have also demonstrated that oxidized SLPI does not inhibit LPS-induced NF-κB activation, unlike wild-type SLPI, indicating that the ability of

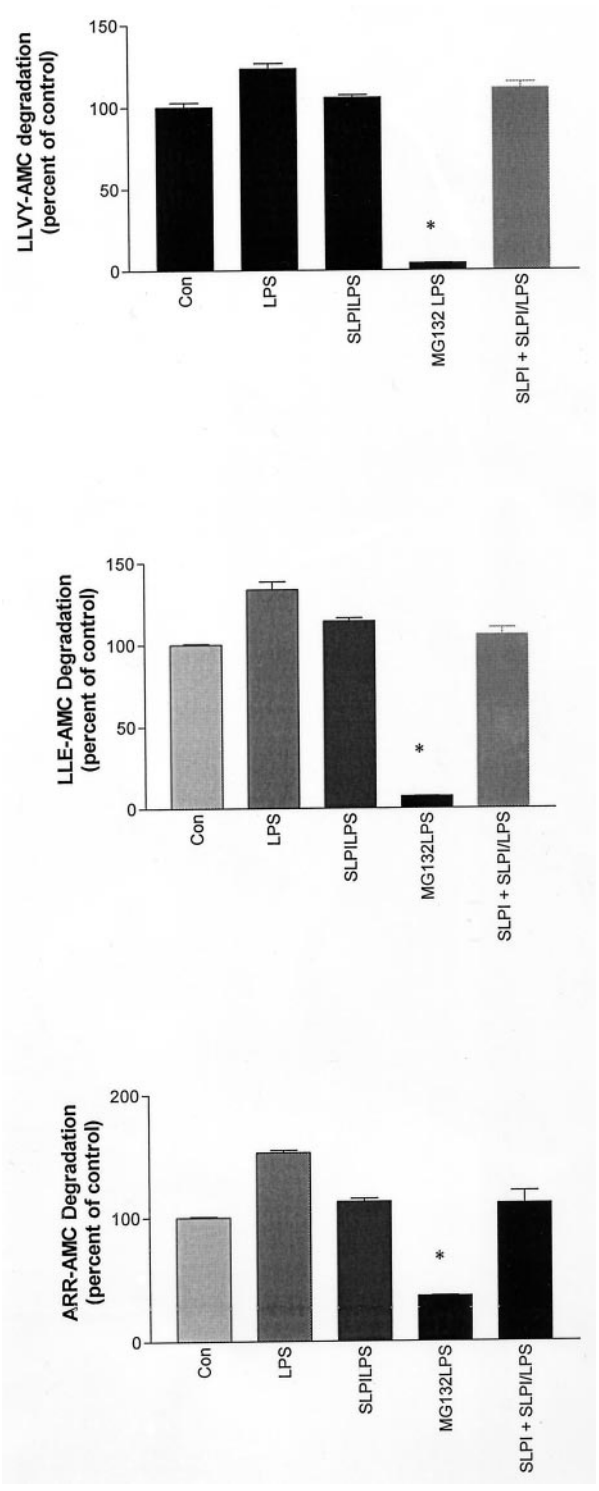

FIG. 4. **SLPI does not inhibit 20 S peptidase activities.** Cytoplasmic extracts from cells treated in medium alone, with LPS, or preincubated with SLPI or MG-132 were assayed for 20 S peptidase activity, *i.e.* for chymotrypsin-like activity using Suc-LLVY-AMC, peptidylglutamyl peptide hydrolyzing activity using Z-Leu-Leu-Glu-AMC, and for trypsin-like activity using Z-Ala-Arg-Arg-AMC. SLPI was also added to some extracts (previously incubated with SLPI/LPS). Extracts were incubated in buffer (25 mM HEPES, 5 mM EDTA, 0.1% CHAPS, 5 mM ATP, pH 7.5 with 2 mM DTT) for 30 min at 37 °C, and fluorescence was determined by excitation at 355 nm and emission at 460 nm. SLPI did not inhibit any of the activities significantly, but MG-132 inhibited all three 20 S-related peptidases significantly. Extracts further treated with SLPI did not show a decrease in 20 S proteasomal activities. Data are the mean ± S.E., $n = 3$ with statistical significance indicated (*, analysis of variance, $p < 0.05$).

SLPI to inhibit the LPS signaling pathway may reside in its antiprotease activity. This result suggests that SLPI may be acting to inhibit a protease or proteases involved in LPS activation of monocytic cells. In this regard, a recent study (21) has shown that another serine protease inhibitor, antithrombin III, inhibits NF-κB activation by LPS.

Apart from the antiprotease and anti-inflammatory properties of SLPI, this multifaceted molecule also possesses antibacterial properties and has been demonstrated to have *in vitro* antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* (22). Another microbicidal peptide, PR-39, has recently been shown to inhibit TNFα-induced IκBα degradation by the ubiquitin-proteasome pathway by binding to the α7 subunit of the 26 S proteasome (23). Although SLPI and PR39 do not share sequence homology, they do share a number of common features including (i) a high positive charge, (ii) a large number of proline residues, (iii) bactericidal activity, and (iv) inducibility of expression by LPS. Therefore, it is possible that SLPI acts similarly to PR39 by entering the cell and inhibiting the ubiquitin-proteasome pathway and thereby inhibiting LPS-induced degradation of the regulatory proteins IRAK, IκBα, and IκBβ and subsequently preventing NF-κB activation. Previous studies (10) have shown that addition of SLPI to monocytes results in SLPI binding to the cell and subsequent detection in cell lysates indicating that SLPI may enter cells to effect its action.

In conclusion, the evidence presented in this study shows that SLPI inhibits LPS-induced NF-κB activation in monocytic cells in a manner that is dependent on its antiprotease activity. Due to its high concentration in the (upper) respiratory tract, the ability of SLPI to inhibit LPS activation of inflammatory cells may be important in disease states such as cystic fibrosis, pneumonia, and acute respiratory distress syndrome. In a previous study (8) we have shown that SLPI is susceptible to proteolytic cleavage by members of the elastolytic cathepsin family, including cathepsins B, L, and S, resulting in the diminution of the antiprotease activity of SLPI. Proteolysis of SLPI may not only impair its anti-elastase activity but also its anti-inflammatory activity. Therefore, oxidation or proteolysis of SLPI during infection or inflammation on the respiratory surface may result in uncontrolled protease activity, generation of greater amounts of pro-inflammatory mediators, and subsequent lung damage.

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J. Biol. Chem. 2002, 277:33648-33653.
doi: 10.1074/jbc.M203710200 originally published online June 25, 2002
