Distinct and Additive Effects of Elastase and Endotoxin on Expression of α₁ Proteinase Inhibitor in Mononuclear Phagocytes*

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Expression of α₁ proteinase inhibitor (α₁-PI) in human mononuclear phagocytes may provide a local mechanism for inactivation of serine proteases at sites of tissue injury, thereby preventing incipient damage to surrounding tissue and allowing for orderly initiation of repair. We have previously shown that serine (neutrophilic or pancreatic) elastase and lipopolysaccharide (LPS) each mediate an increase in the expression of α₁-PI in human peripheral blood monocytes and bronchoalveolar macrophages. In this study we demonstrate that elastase and LPS have an additive positive regulatory effect on α₁-PI expression. Distinct pretranslational and translational mechanisms of action for elastase and LPS, respectively, account for the additive effect. The possibility that translational regulation of α₁-PI by LPS involves a mechanism analogous to that of the yeast gene GCN4 during amino acid starvation and that of the human ferritin gene in response to iron is discussed.

At sites of tissue injury or inflammation, proteolytic enzymes are released from neutrophils, platelets, activation products of the complement, coagulation and fibrinolytic systems, damaged tissue, and from invading microorganisms. These proteases may directly contribute to the host inflammatory response or prime macrophages for a more effective oxidative metabolic response to microorganisms or tumor cells (1). However, they may also produce greater incidental tissue destruction. Macrophages are now known to synthesize and secrete protease inhibitors including α₁ proteinase inhibitor (α₁-PI)¹ (2, 3), C1 inhibitor (4, 5), plasminogen activator-inhibitor (6), α₂-macroglobulin (7), and collagenase inhibitor (8). Such protease inhibitors, produced locally by macrophages at sites of tissue injury/inflammation, may act to limit tissue damage and facilitate repair.

One of these protease inhibitors, α₁-PI, is a 55-kDa glycoprotein that rapidly inactivates neutrophil elastase (reviewed inRefs. 9, 10). It is a prototypic suicidal serine protease inhibitor (reviewed in Ref. 11) and shares molecular structural characteristics with other members of the serpin supergene family that include antithrombin III (12), α₁-antichymotrypsin (13), C1 inhibitor (14, 15), α₂-antiplasmin (16), protein C inhibitor (17), plasminogen activator-inhibitor II (18, 19), ovalbumin (12), and angiotensinogen (20). Although serum α₁-PI is probably predominantly derived from liver (21, 22), expression of this inhibitor in extrahepatic sites by monocytes and macrophages has several interesting, if not important, characteristics. First, transcription of the α₁-PI gene in macrophages starts at a promoter located ~2 kilobases upstream from the promoter which directs α₁-PI gene transcription in hepatoma cells (23, 24). Although an identical α₁-PI molecule is synthesized and secreted, the longer macrophage α₁-PI mRNA has two short open reading frames and, thus, might contain additional coding capacity (23). Second, expression of α₁-PI in human monocytes and macrophages is subject to tissue-specific regulation by the enzyme it inhibits, neutrophil elastase (25), and by bacterial lipopolysaccharide (LPS) (26). The regulatory effects of elastase and LPS on α₁-PI gene expression are distinct; the effect of elastase is rapid, becoming evident within 3-4 h, whereas that of LPS is not observed until 6-8 h of incubation. The effect of elastase, but not that of LPS, is neutralized by the serine protease inhibitor diisopropylfluorophosphate and antibody to human neutrophil elastase (25). The effect of LPS, but not that of elastase, is neutralized by monoclonal antibody to the lipid A moiety of LPS (25, 26). The effect of elastase results from a specific increase in steady-state levels of α₁-PI mRNA, whereas LPS mediates a 5-9-fold increase in synthesis of α₁-PI with minimal, or no change, in α₁-PI mRNA levels. Since both of these substances are likely to be present at sites of tissue injury/inflammation, and since each has an effect on macrophage α₁-PI expression at a distinct biosynthetic level, we examined the possibility of additive or synergistic actions for elastase and LPS in modulating macrophage α₁-PI expression.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium without methionine was purchased from GIBCO. Medium 199 and Hank’s balanced salt solution were purchased from Microbiological Associates. Fetal calf serum, human AB serum, L-glutamine, penicillin-streptomycin were from Flow Laboratories. [18S]Methionine (~1000 Ci/mmol) was obtained from ICN Radiochemicals (Trans 3S label) and [32P]deoxyctydine triphosphate (~3000 Ci/mmol) and 4C methylated protein standards were obtained from Amersham Corp. Other reagents included IgG-Sorb from Enzyme Center, caesium chloride from Bethesda Research Laboratories, guanidine isothiocyanate from Fluka, sodium N-laurylsarcosinate from ICN Pharmaceuticals. LPS extracted from Escherichia coli serotype 0111:B4 and porcine pancreatic elastase were purchased from Sigma. Preparations of human neutrophil elastase were obtained from Elastin Products and provided by Dr. James Travis, Athens, GA. Rabbit anti-human α₁-PI was from Dako, goat

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The abbreviations used are: α₁-PI, α₁ proteinase inhibitor; LPS, lipopolysaccharide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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anti-human factor B from Atlantic Antibodies, and sheep anti-human C2 from Miles Laboratories.

Cell Culture—Confluent monolayers of human blood monocytes from normal PiM individuals were established by a modification of the previously described technique (27). White blood cells from leukapheresis packs were further purified by dextran-EDTA sedimentation and monocytes then purified by adherence of the cells in the buffy coat to Primaria® tissue culture plates or dishes in 15% human AB serum. Unless otherwise specified, experiments were done after 24 h in culture. Monolayers were rinsed and then incubated in serum-free Medium 199 alone or supplemented with elastase, LPS, or both. The presence of LPS in medium or supplements was monitored by a limulus amebocyte lysate assay (Associates of Cape Cod). Biosynthetic Labeling—Confluent monolayers were rinsed and incubated at 37 °C in the presence of methionine-free medium containing [35S]methionine, 500 mCi/ml. To determine the rate of synthesis of α1-PI or control secretory proteins, cells were subjected to a short pulse interval (30 min) and radiolabeled proteins detected in the cell lysates. Methods for solubilization of cells and clarification of cell lysates after labeling have been described (2). Total protein synthesis was estimated by trichloroacetic acid precipitation of aliquots of cell lysates (28). Primary or total translation products were generated in a commercial rabbit reticulocyte lysate translation system (Du Pont-New England Nuclear). 

Immunoprecipitation and SDS-PAGE—Aliquots of cell lysate were incubated overnight at 4 °C in 1% Triton X-100, 1.0% SDS, 0.5% deoxycholic acid, with excess antibody. Immune complexes were precipitated with excess formalin-fixed staphylococci-bearing protein A-Sepharose beads, washed, released by boiling in sample buffer, and applied to 9.0% SDS-PAGE under reducing conditions, as described by Laemmli (29). 14C-Methylated molecular size markers (200,000, 92,500, 68,000, 46,000, 30,000, and 17,000 Da) were included on all gels. After electrophoresis, gels were stained with Coomassie Brilliant Blue, destained, impregnated with 2,5-diphenyloxazole (EN3HANCE, Du Pont-New England Nuclear), and dried for fluorography on XAR x-ray film (Eastman Kodak). An LKB laser densitometer 2222 Ultrascan XL was used for scanning of fluorograms. Detection of RNA by RNA Blot Analysis—Total cellular RNA was isolated from adherent monolayers and monocytes by guanidine isothiocyanate extraction and ethanol precipitation (30). For specific experiments RNA preparations were extracted with phenol-chloroform and then subjected to ethanol precipitation. RNA was quantified by absorbance at 260 nm and solubilized for agarose-formaldehyde gel electrophoresis and transfer to nitrocellulose filters (31). Filters were then hybridized with 3P-labeled cDNA specific for human α1-PI (29) and human factor H (32).

RESULTS

Elastase and LPS Have Additive Effects under Submaximal Conditions—The collaborative effects of elastase and LPS were first examined at different concentrations for submaximal time intervals. Confluent monolayers of monocytes were incubated for 16 h in serum-free control medium, medium supplemented with elastase alone, or LPS alone. Separate monolayers were first incubated for 8 h in elastase, rinsed thoroughly, and then incubated for an additional 8 h in control medium, supplemented with LPS, or elastase alone. Elastase mediated a concentration-dependent increase in synthesis of α1-PI, reaching 2.9-fold at a concentration of 0.2 units/ml, or 50 ng/ml (Fig. 1). At higher concentrations there was a plateau, or even slight decrease, in the effect of elastase. LPS also had a concentration-dependent effect, reaching 3.0-fold at the highest concentration. Monocytes first primed with elastase and then incubated with LPS increased synthesis of α1-PI to 4.8-fold (Fig. 1). At each concentration of elastase examined, LPS elicited an additive effect on synthesis of α1-PI. In monocytes incubated with elastase and LPS, there was a plateau, or decline, in the additive effect when elastase concentrations exceeded 0.2 units/ml. Results were similar when monocytes were primed with LPS, rinsed thoroughly, and then incubated with elastase (data not shown). In either case, there was no significant change in the synthesis of a control secretory protein, the second component of complement (C2), as determined by sequential immunoprecipitation of the same radiolabeled cell lysates (data not shown).

The additive effect of elastase and LPS was also evident during shorter intervals of incubation. There was a 2-fold increase in rate of α1-PI synthesis in monocytes incubated for 12 h in medium supplemented with pancreatic elastase (lanes 2, 0.0002 units/ml; lane 3, 0.002 units/ml; lane 4, 0.02 units/ml; lane 5, 0.2 units/ml; lane 6, 2.0 units/ml), or control medium supplemented with LPS (lanes 7, 0.5 ng/ml; lane 8, 5 ng/ml; lane 9, 50 ng/ml; lane 10, 500 ng/ml). Separate monolayers of monocytes were first incubated for 8 h with elastase (lanes 11 and 14, 0.0002 units/ml; lanes 12 and 15, 0.02 units/ml; lanes 13 and 16, 2.0 units/ml), rinsed thoroughly and incubated for an additional 8 h with LPS (lanes 11–13, 50 ng/ml; lanes 14–16, 500 ng/ml). Monolayers were then rinsed and subjected to pulse radiolabeling for 30 min. Newly synthesized, radiolabeled α1-PI was assayed by subjecting the cell lysates to immunoprecipitation, SDS-PAGE followed by fluorography. Molecular mass markers are indicated at the right margin. The magnitude of the increase in α1-PI synthesis is shown in the lower panel. Each point represents the average of two separate experiments including data shown in the upper panel.

The additive effect on synthesis of α1-PI was also demonstrated in monocytes incubated with both elastase and LPS for maximal time intervals (24 h) at maximal concentrations (0.2 units/ml and 500 ng/ml, respectively). The effects of porcine pancreatic elastase and human neutrophil elastase were interchangeable (data not shown).

LPS Mediates a Selective Increase in the Efficiency of Translation of α1-PI mRNA—Total cellular RNA was isolated from blood monocytes that had been incubated for 24 h in serum-free control medium and control medium supplemented with

**FIG. 1. The effect of elastase and LPS on synthesis of α1-PI in peripheral blood monocytes.** After 24 h in culture monocytes were incubated for 16 h in serum-free control medium alone (lane 1), control medium supplemented with pancreatic elastase (lane 2, 0.0002 units/ml; lane 3, 0.0002 units/ml; lane 4, 0.02 units/ml; lane 5, 0.2 units/ml; lane 6, 2.0 units/ml), or control medium supplemented with LPS (lane 7, 0.5 ng/ml; lane 8, 5 ng/ml; lane 9, 50 ng/ml; lane 10, 500 ng/ml). Separated monolayers of monocytes were first incubated for 8 h with elastase (lanes 11 and 14, 0.0002 units/ml; lanes 12 and 15, 0.02 units/ml; lanes 13 and 16, 2.0 units/ml), rinsed thoroughly and incubated for an additional 8 h with LPS (lanes 11–13, 50 ng/ml; lanes 14–16, 500 ng/ml). Monolayers were then rinsed and subjected to pulse radiolabeling for 30 min. Newly synthesized, radiolabeled α1-PI was assayed by subjecting the cell lysates to immunoprecipitation, SDS-PAGE followed by fluorography. Molecular mass markers are indicated at the right margin. The magnitude of the increase in α1-PI synthesis is shown in the lower panel. Each point represents the average of two separate experiments including data shown in the upper panel.
FIG. 2. The effect of short-term incubation with elastase and LPS on the synthesis of $\alpha_1$-PI in monocytes. After 24 h in culture monocytes were incubated for 12 h in serum-free control medium alone (lane 1), control medium supplemented with LPS 500 ng/ml (lane 2), or control medium supplemented with elastase, 0.2 units/ml (lane 8). Separate monolayers were first incubated for 6 h in culture medium supplemented with LPS (lanes 3, 0.05 ng/ml; lane 4, 0.5 ng/ml; lane 5, 5 ng/ml; lane 6, 50 ng/ml; lane 7, 500 ng/ml), rinsed thoroughly and then incubated for another 6 h in medium supplemented with elastase 0.2 units/ml (lanes 3–7). Other monolayers were first incubated in elastase (lane 9, 0.002 units/ml; lane 10, 0.002 units/ml; lane 11, 0.02 units/ml; lane 12, 0.2 units/ml; lane 13, 2 units/ml), rinsed thoroughly and then incubated for another 6 h in medium supplemented with LPS 500 ng/ml (lanes 9–13). Monolayers were rinsed, pulse-radiolabeled and cell lysates subjected to the analytical system as described in Fig. 1. Relative densitometric units for this experiment: lane 1, 0.0645; lane 2, 0.1022; lane 3, 0.1023; lane 4, 0.0725; lane 5, 0.1348; lane 6, 0.1684; lane 7, 0.1558; lane 8, 0.1245; lane 9, 0.1575; lane 10, 0.1607; lane 11, 0.2076; lane 12, 0.2382; lane 13, 0.1949.

FIG. 3. The effect of LPS on steady-state levels of $\alpha_1$-PI mRNA and cell-free synthesis of $\alpha_1$-PI. After 24 h in culture, monocytes were incubated for 24 h in control medium or medium supplemented with LPS (10 ng/ml). Total cellular RNA was isolated and 10 μg of each sample subjected to RNA blot analysis (panel a). There was no significant difference in the intensity of ethidium bromide-stained ribosomal RNA bands in these two lanes. The same cDNA. RNA from the same preparations was subjected to cell-free translation in a commercial rabbit reticulocyte lysate system (panel b). There was no significant difference in the intensity of ethidium bromide-stained ribosomal RNA bands in these two lanes. The same cDNA. RNA from the same preparations was subjected to cell-free translation in a commercial rabbit reticulocyte lysate system (panel b). There was no significant difference in the intensity of ethidium bromide-stained ribosomal RNA bands in these two lanes.

LPS. This RNA was first subjected to RNA blot analysis (Fig. 3, panel a). LPS did not significantly affect steady-state levels of $\alpha_1$-PI mRNA but mediated a 5-fold increase in complement factor B mRNA levels. The same RNA preparations were used to program a rabbit reticulocyte lysate translation system (panel b). An approximately 46-kDa primary translation product was recognized by antibody to $\alpha_1$-PI. It is not known whether a slower migrating, approximately 48-kDa, radiolabeled polypeptide is a distinct primary translation product for $\alpha_1$-PI, a modified form of the predominant 46-kDa primary translational product, or a non-specific product of immunoprecipitation. In contrast to the minimal changes in steady-state levels of $\alpha_1$-PI mRNA, there was a marked increase in the cell-free synthesis of $\alpha_1$-PI mediated by LPS (panel b). The effect of LPS on the translational efficiency of $\alpha_1$-PI mRNA was demonstrated at two different concentrations of RNA input. The increase in cell-free synthesis of factor B (panel b) corresponded to changes in factor B mRNA levels (panel a). There was no significant difference in acid-precipitable protein (not shown) or total products of in vitro translation (panel b) for these experiments. The selective increase in translational efficiency of $\alpha_1$-PI mRNA from LPS-activated monocytes was also evident when RNA was completely deproteinized by phenol treatment (Table I).

The Additive Effect Involves Distinct Pretranslational and Translational Mechanisms of Action—Total cellular RNA was isolated from monocytes incubated for 16 h with elastase alone or LPS alone, or from monocytes first incubated with elastase then LPS. This RNA was subjected to RNA blot analysis. After a 16-h incubation elastase-mediated a significant increase in steady-state levels of $\alpha_1$-PI mRNA (Fig. 4, lane 3) but LPS had no effect on $\alpha_1$-PI mRNA levels (lanes 8 and 9). There was no difference in steady-state levels of $\alpha_1$-PI mRNA in monocytes incubated with elastase alone as compared to monocytes first incubated in elastase and then LPS (lanes 4–7). The changes in $\alpha_1$-PI mRNA levels were specific in that there were no significant differences in the total product of in vitro translation programmed with the same RNA preparations (Fig. 4, lower panel). These results suggest that the additive effect of elastase and LPS involves distinct pretranslational and translational mechanisms of action. The same RNA was then subjected to in vitro translation to assay the specific activity of $\alpha_1$-PI mRNA (Fig. 5). Equivalent amounts of total cellular RNA were used to program the rabbit reticulocyte lysate translation system. There was a significant increase in the accumulation of $\alpha_1$-PI-specific primary translation product mediated by LPS alone (lane 4). This likely represents an increase in translation since there was no significant change when the amount of total cellular RNA used in translation was lowered to account for that effect (not shown).

The additive effect of elastase and LPS was evident at the level of translation since there was an ~4- to 6-fold increase in accumulation of the $\alpha_1$-PI primary translation product using an equivalent amount of RNA from elastase-treated monocytes (lane 2). This probably reflects the increase in $\alpha_1$-PI mRNA levels since there was no significant increase when the amount of total cellular RNA used in translation was lowered to account for that effect (not shown). The additive effect of elastase and LPS was evident at the level of translation since there was an ~4- to 6-fold increase in accumulation of the $\alpha_1$-PI primary translation product under such conditions (lane 3). The effects of elastase and LPS on the efficiency of translation of monocytes-derived $\alpha_1$-PI

<table>
<thead>
<tr>
<th>RNA</th>
<th>Primary translation product</th>
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<tbody>
<tr>
<td></td>
<td>−Phenol</td>
</tr>
<tr>
<td>Control &amp;</td>
<td>1.00</td>
</tr>
<tr>
<td>LPS*</td>
<td>1.55</td>
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</tbody>
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* Monocytes were incubated in serum-free control medium or medium supplemented with LPS 10 ng/ml for 24 h, exactly as in Fig. 3.
mRNA were similar for RNA subjected to deproteinization by phenol extraction (Table II).

**DISCUSSION**

These results indicate that elastase and LPS mediate an additive increase in the expression of the elastase inhibitor \(\alpha_1\)-PI in human mononuclear phagocytes. Distinct pretranslational and translational mechanisms of action for elastase and LPS, respectively, account for the additive effect. Previous studies have suggested that LPS regulates the expression of specific genes in macrophages by transcriptional and/or post-transcriptional effects (33–36). Where it has been studied in detail, the post-transcriptional effects of LPS are attributable to stabilization of short-lived mRNAs (34, 37). These sequences have recently been shown to control the translation of the GCN4 gene product both under repressor proteins to \(\alpha_1\)-PI mRNA. It is unusual for eukaryotic mRNAs to have a decrease in binding of repressor proteins to \(\alpha_1\)-PI mRNA. Such a mechanism is thought to be responsible for control of the expression of a subclass of mRNAs in fibroblasts (44).

TABLE II

<table>
<thead>
<tr>
<th>RNA</th>
<th>Primary translation product</th>
<th>−Phenol</th>
<th>+Phenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control(a)</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Elastase(a)</td>
<td>1.96</td>
<td>1.97</td>
<td>2.47</td>
</tr>
<tr>
<td>Elastase + LPS(a)</td>
<td>2.01</td>
<td>5.85</td>
<td>4.26</td>
</tr>
<tr>
<td>LPS(a)</td>
<td>1.08</td>
<td>3.30</td>
<td>2.89</td>
</tr>
</tbody>
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\(a\)Monocytes were incubated in serum-free control medium, medium supplemented with elastase 0.2 units/ml for 16 h, medium supplemented with LPS 50 ng/ml for 16 h, medium supplemented with elastase 0.2 units/ml plus LPS 50 ng/ml for 8 h, and then LPS 50 ng/ml for 8 h, exactly as in Fig. 5.

start site for transcription of \(\alpha_1\)-PI mRNA in macrophages, ~2 kilobases upstream from that governing transcription of \(\alpha_1\)-PI mRNA in hepatocytes (23, 24). Within the 2 kilobases of DNA between the two potential transcriptional start sites are two short segments of open reading frame with initiation codons (23). It is unusual for eukaryotic mRNAs to have multiple upstream AUG codons. There are four short open reading frames in the 5’-untranslated region of the yeast GCN4 mRNA, each complete with initiation and termination codon (38, 39). These sequences have recently been shown to control the translation of the GCN4 gene product both under basal conditions and in response to amino acid starvation (40). A sequence within the 5’-untranslated region of the human ferritin gene is responsible for translation regulation of that gene by iron (41–43). It will be of great interest to determine whether the open reading frames in the 5’-flanking region of \(\alpha_1\)-PI gene are involved in control of its translation during LPS-activated states. An alternative explanation for the effect of LPS on the translational efficiency of \(\alpha_1\)-PI mRNA is a decrease in expression of “repressor” proteins or a decrease in binding of repressor proteins to \(\alpha_1\)-PI mRNA. Such a mechanism is thought to be responsible for control of the expression of a subclass of mRNAs in fibroblasts (44).

![Graphical representation](image-url)
Finally, it is possible that the effect of LPS on translation of $\alpha$-PI mRNA involves a change in RNA folding (45).

LPS collaborates with several other mediators in activation of monocytes and macrophages. In particular, LPS and interferon-γ have synergistic effects on macrophage-derived tumor cytotoxicity (46–48) expression of cachectin/tumor necrosis factor (33) and release of interleukin-1 (49). In these cases interferon is thought to have a permissive, or priming, effect on macrophage responses to LPS. For other macrophage functions LPS and interferon-γ may have antagonistic effects, including respiratory burst capacity (50) and expression of granulocyte macrophage-colony stimulating factor (34). Elastase may also collaborate with other substances in activation of macrophages. Speer et al. (1) have shown that human monocytes, after 3 days in culture, may be primed by neutrophil elastase for enhanced release of toxic oxygen metabolites in response to phorbol esters or opsonized zymosan.

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REFERENCES