LIPOPOLYSACCHARIDE MODULATES THE EXPRESSION OF α_1 PROTEINASE INHIBITOR AND OTHER SERINE PROTEINASE INHIBITORS IN HUMAN MONOCYTES AND MACROPHAGES

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At sites of inflammation or tissue injury, proteolytic enzymes are released by neutrophils, platelets, damaged tissue, and activation products of the complement, coagulation, and fibrinolytic pathways. These proteinases must ultimately be inactivated to prevent incidental destruction of surrounding uninvolved tissue and to ensure the orderly initiation of tissue repair. It has recently been recognized that protease inhibitors may be produced by macrophages as well as by liver and, therein, provide both local and remote mechanisms for proteinase inactivation at sites of tissue injury. Human monocytes and macrophages synthesize and secrete α_1 proteinase inhibitor (PI)[1] (1, 2), Cl inhibitor (3, 4), plasminogen-activator inhibitor (5), α_2 macroglobulin (6), and collagenase inhibitor (7).

α_1 PI is a 55-kD glycoprotein that constitutes the principle serum inhibitor of neutrophil elastase. It is encoded by an ~10-kb gene on human chromosome 14 (8, 9). It is considered a member of a supergene family that includes antithrombin III, ovalbumin (10), angiotensinogen (11), α_2 antiplasmin (12), α_1 antichymotrypsin (13), protein C inhibitor (14) and C1 inhibitor (15, 16). These genes bear 30–40% primary structural homology and have a number of residues around the active inhibitory region that are highly conserved. Each one of these serpins is characterized by a specificity for inhibition of individual serine proteinases, although each is also able to inhibit other serine proteinases less effectively (reviewed in reference 17).

There is considerable polymorphic variation of α_1 PI. One variant allele, PiZ, is associated with a severe reduction in serum concentrations of functionally active α_1 PI. This deficiency is often associated with premature development of pulmonary emphysema, and 15–20% of PiZZ individuals are affected by pro-

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[1] *Abbreviation used in this paper:* PI, proteinase inhibitor.

gressive liver disease (reviewed in references 18, 19). A number of studies have directly demonstrated (2, 20–22) a selective defect in secretion of α_1 PI in PiZZ individuals, probably affecting the transport of α_1 PI from the endoplasmic reticulum to the Golgi apparatus. The defect is expressed in liver cells and cells of mononuclear phagocyte lineage (2).

Plasma concentrations of α_1 PI increase three- to fourfold during acute inflammation or tissue injury (23). A number of other human hepatic acute-phase reactants have now been shown (24–25) to be regulated in human hepatoma cells (HepG2 and Hep3B) by the monokines IL-1 and cachectin/TNF. The rate of synthesis of α_1 PI in HepG2 and Hep3B cells is not affected by IL-1, TNF (25), or by supernatants of LPS-stimulated peripheral blood mononuclear cells (26). Conversely, significant increases in expression of α_1 PI have been demonstrated in human monocytes and macrophages. A product of mitogen-stimulated $T4^+$ lymphocytes (27) mediates a 2.0–2.5-fold increase and neutrophil elastase (28) mediates a 3.5–8-fold increase in steady-state levels of α_1 PI mRNA and rate of synthesis of α_1 PI in cultured extrahepatic mononuclear phagocytes. In this study we examined the effect of inflammatory activation by a bacterial product, LPS, on expression of the α_1 PI gene in macrophages from normal and homozygous PiZZ α_1 PI-deficient individuals.

Materials and Methods

DME and DME lacking methionine were purchased from Gibco Laboratories, Grand Island, NY. HBSS and Medium 199 (M199) were purchased from Microbiological Associates, Walkersville, MD. FCS, L-glutamine, and penicillin-streptomycin were from Flow Laboratories, Inc., McLean, VA. [^{35}S]Methionine (specific radioactivity ~1,000 Ci/mmol) and [^{32}P]deoxycytidine triphosphate (specific radioactivity ~3,000 Ci/mmol) were obtained from New England Nuclear, Boston, MA, and [^{14}C]methylated protein standards were from Amersham Corp., Arlington Heights, IL. Other reagents included IgG-Sorb from Enzyme Center, Cambridge, MA; cesium chloride from Bethesda Research Laboratories, Gaithersburg, MD; guanidine isothiocyanate from Fluka AG, Buchs, Switzerland; and sodium-*N*-laurylsarcosinate from ICN Pharmaceuticals, Inc., Irvine, CA. Goat anti–human factor B, goat anti–human C3, goat anti–human C1 inhibitor were purchased from Atlantic Antibodies, Scarborough, ME; rabbit anti–human α_1 PI and rabbit anti–human α_2 macroglobulin were from Dako Corp., Santa Barbara, CA; and sheep anti–human C2 came from Miles Laboratories Inc., Naperville, IL. Goat anti–human neutrophil elastase was provided by James Travis, University of Georgia, Athens, GA. LPS preparations extracted from two different strains of *Escherichia coli* (serotypes 0111:B4 and 026:B6), each extracted by two different procedures (TCA precipitation and Westphal phenolic extraction) were purchased from Sigma Chemical Co. *E. coli* 0113 LPS was also purchased from Associates of Cape Cod, Inc., Woods Hole, MA. mAb to the lipid A moiety of LPS (8A1 [29]) was kindly provided by Dr. Warren C. Bogard, Centocor Malvern, PA.

Cell Culture. Confluent monolayers of human peripheral blood monocytes from 24 normal PiMM and 3 symptomatic PiZZ individuals (as defined by IEF, serum levels, and family studies) were established by adherence of dextran-purified leukocytes on siliconized glass as previously described (30). Bronchoalveolar macrophages were obtained from sterile saline bronchial lavage. After centrifugation and washing, cells were allowed to adhere to siliconized glass cover slips (31). HepG2 and Hep3B cells were maintained in culture as previously described (32). Murine L cells transfected with the cloned human α_1 PI gene (TfAT2) were maintained in selective medium. The TfAT2 cell line was derived by cotransfection of murine Ltk^- cells with HSV thymidine kinase DNA and a genomic DNA clone bearing the entire human α_1 PI gene and 5–6 kb of 5′ and 3′ flanking regions

(AT73 [33], kindly provided by Professor R. Cortese, Heidelberg, Federal Republic of Germany) by calcium phosphate precipitation (34). Synthesis and secretion of α_1 PI was demonstrated in this cell line, but not in the parent untransfected Ltk$^-$ cell line (28).

LPS contamination was detected and quantified by limulus amebocyte lysate assay (Associates of Cape Cod, Inc.). The LPS concentration of the cell culture before the addition of exogenous LPS was <10 pg/ml, when detectable.

Biosynthetic Labeling. Confluent monolayers were rinsed and incubated at 37°C in the presence of methionine-free medium containing [^{35}S]methionine, 500 μCi/ml (pulse period). To determine the rate of synthesis of α_1 PI or control secretory proteins, cells were subjected to a short pulse interval (30 min), and radiolabeled proteins were detected in the cell lysate alone. To determine the rate of secretion of α_1 PI, cells were subjected to a pulse period of 30 min, rinsed, and incubated in serum-free medium containing an excess of unlabeled methionine (chase period). Radiolabeled α_1 PI was detected in cell culture fluid and cell lysates at specified intervals of the chase period. To determine the accumulation of α_1 PI in the cell culture fluid, cells were subjected to a long pulse interval (3 h) and radiolabeled α_1 PI was identified in cell culture fluid alone. Methods for solubilization of cells and clarification of cell lysates after labeling have been described (1). Total protein synthesis was estimated by TCA precipitation of aliquots of cell lysates and culture fluid (35).

Immunoprecipitation and SDS-PAGE. Aliquots of cell lysate or medium 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, washed, released by boiling in sample buffer, and applied to 9.0% SDS-PAGE under reducing conditions as described by Laemmli (36). ^{14}C-methylated molecular size markers (200,000; 92,500; 68,000; 46,000; 30,000; and 17,000 mol wt) were included on all gels. After electrophoresis, gels were stained in Coomassie Brilliant Blue, destained, impregnated with 2,5-diphenyloxazole (EN^{3}HANCE, New England Nuclear), and dried for fluorography on XAR x-ray film (Eastman Kodak Co., Rochester, NY). LKB Instruments, Inc. Houston, TX 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 of monocytes and macrophages by guanidine isothiocyanate extraction and ethanol precipitation (37). RNA was quantified by absorbance at 260 nm and solubilized for agarose-formaldehyde gel electrophoresis and transfer to nitrocellulose filters (38). Filters were then hybridized with ^{32}P-labeled cDNA specific for human α_1 PI (39), human factor B (40), human C3 (41), and human α_2 macroglobulin (kindly provided by Dr. B. F. Tack, Scripps Clinic and Research Institute, La Jolla, CA).

Results

LPS Mediates an Increase in the Rate of Synthesis of α_1 PI in Human Blood Monocytes and Bronchoalveolar Macrophages. Monolayers of peripheral blood monocytes after 24 h in culture were incubated for an additional 24 h in control serum-free medium or medium supplemented with LPS (Fig. 1, *left*). LPS mediates a concentration-dependent increase in the rate of synthesis of α_1 PI. The effect of LPS is evident at concentrations of LPS as low as 1 ng/ml. At the highest concentration tested (1 μg/ml), the rate of α_1 PI synthesis increased 8.7-fold. LPS from three different strains of *E. coli*, each prepared by two different extraction procedures, have the same effect on synthesis of α_1 PI (data not shown). Accumulation of α_1 PI in the intracellular contents and cell culture fluid after a long interval of pulse radiolabeling increased 5.0- and 4.6-fold, respectively (Fig. 1, *right*), suggesting that LPS does not have a significant effect on the rate of posttranslational processing or secretion of newly synthesized α_1 PI. Pulse-chase experiments, described below, also demonstrate that LPS affects the rate

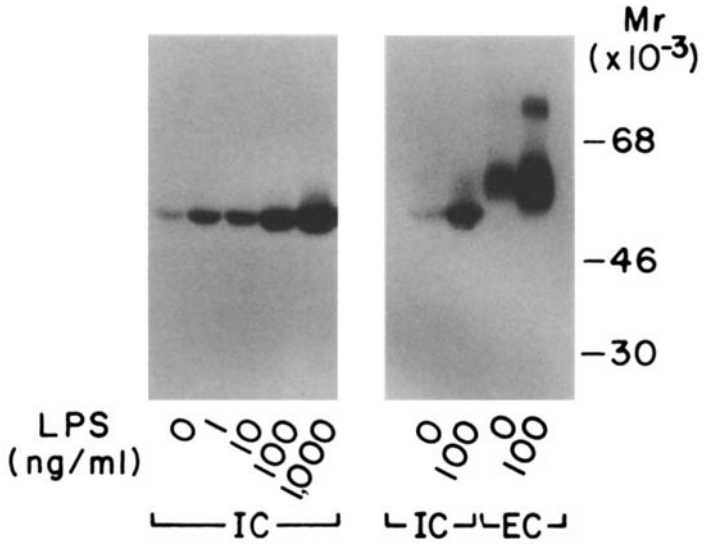

FIGURE 1. Effect of LPS on synthesis and secretion of α_1 PI in peripheral blood monocytes. After 24 h in culture, monocytes were incubated for 24 h in serum-free medium alone (0) or supplemented with LPS in the specified concentrations. Cells were then subjected to pulse radiolabeling for 30 min (*left*) or 3 h (*right*). α_1 PI was detected in cell lysates (*left*; *right*, IC) or culture fluid (EC). Molecular mass markers are indicated in the right margin.

of synthesis of α_1 PI but not the rate of its secretion. The effect of LPS results in an increase of similar magnitude in the accumulation of native 55-kD α_1 PI and a 75-kD form of α_1 PI in complex with an endogenous monocyte serine elastase (1, 42, 43) (Fig. 1, *right*). Activation of monocytes by LPS, therefore, results in an increase in functional activity of α_1 PI, as defined by the capacity to form a stable complex with serine elastase, as well as an increase in rate of synthesis of α_1 PI (Fig. 1, *left*). Moreover, there is an increase in functional activity of α_1 PI despite the potential for inactivation by oxygen radicals (44), cysteine proteases (45), or metalloenzymes (46), which may also be released by LPS-activated monocytes. Conversely, the rate of synthesis of elastase in monocytes is not affected by LPS (Fig. 2).

The effect of LPS is time dependent (Fig. 3). Rate of synthesis of α_1 PI increases within 8 h. The effect of LPS is still evident after 24 h.

LPS has a similar effect on α_1 PI expression in blood monocytes and tissue macrophages. The rate of synthesis of α_1 PI increases 4.8–7.2-fold in monocytes incubated with LPS after 1, 5, and 10 d in culture (Fig. 4), despite the specific decrease in expression of α_1 PI that ordinarily accompanies in vitro maturation of monocytes (1, 27). The rate of synthesis of α_1 PI increases 4.5-fold in bronchoalveolar macrophages incubated with LPS after 1 d in culture (Fig. 5).

LPS also regulates the expression of other serine protease inhibitors in monocytes and macrophages. The rate of synthesis of C1 inhibitor increases 5-fold in LPS-treated alveolar macrophages, while that of α_2 macroglobulin decreases 3.7-fold (Fig. 5). These effects are specific in that other products of the macrophage increase to a different extent, including complement components C3 (3-fold) and factor B (4.2-fold), or are not affected, as shown by C2 (Fig. 5).

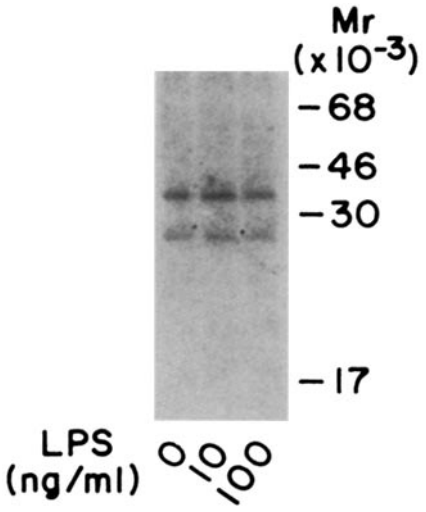

FIGURE 2. Effect of LPS on synthesis of neutrophilic elastase in peripheral blood monocytes. Samples from the experiment shown in Fig. 1, *left*, were immunoprecipitated with goat anti–human neutrophil elastase after immunoprecipitation with anti–human α_1 PI. M_r markers indicated in the right margin.

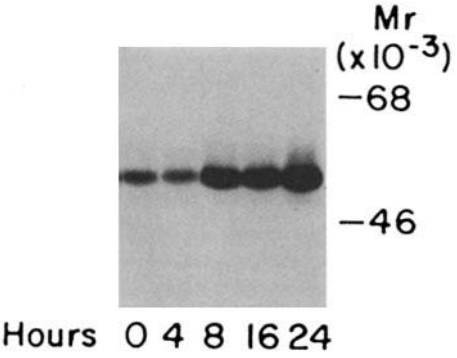

FIGURE 3. The effect of LPS on synthesis of α_1 PI is time dependent. After 24 h in culture monocytes were incubated for 24 h in serum-free medium alone (0) or supplemented with LPS, 100 ng/ml, for the specified time intervals. Cells were then subjected to pulse radiolabeling for 30 min and α_1 PI was detected in cell lysates. M_r markers are indicated in the right margin.

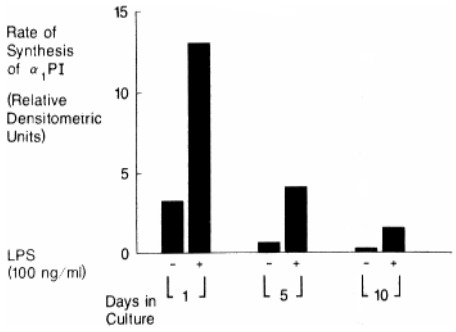

FIGURE 4. LPS regulates the synthesis of α_1 PI in human monocytes during in vitro maturation. Monolayers of monocytes after 1, 5, and 10 d in culture were incubated for 24 h in serum-free medium alone (−) or medium supplemented with LPS, 100 ng/ml. Cells were then subjected to pulse radiolabeling for 60 min and α_1 PI was detected in cell lysates. Results are expressed as relative densitometric units.

The effect of LPS on expression of α_1 PI in mononuclear phagocytes was neutralized by preincubation with mAb to *E. coli* lipid A (Fig. 6). LPS had no effect on the rate of synthesis of α_1 PI in human hepatoma cells HepG2 or Hep3B or in a stable transfected cell line, mouse fibroblast L cells transfected with the human α_1 PI gene (data not shown).

The Effect of LPS on Expression of α_1PI in Mononuclear Phagocytes Involves Both

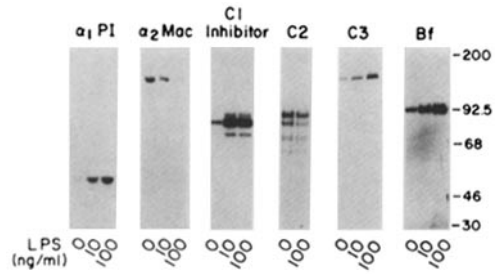

FIGURE 5. Specificity of the response of macrophages to activation by LPS. After 24 h in culture, confluent monolayers of macrophages were incubated for 24 h in serum-free medium (0) or medium supplemented with LPS in the specified concentrations. Cells were then subjected to pulse radiolabeling. α_1 PI, other serine protease inhibitors, and serine proteases were detected in cell lysates by sequential immunoprecipitation. Molecular mass markers are indicated in the right margin.

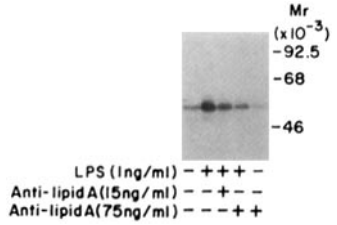

FIGURE 6. Specificity of the effect of LPS on expression of α_1 PI in blood monocytes. After 24 h in culture monocytes were incubated for 8 h in serum-free medium alone, medium supplemented with LPS 1 ng/ml, medium supplemented with LPS 1 ng/ml, and mAb to lipid A (8A1 [29]), 15 ng/ml or 75 ng/ml, or medium supplemented with monoclonal antibody to lipid A, 75 ng/ml alone. Cells were then subjected to pulse radiolabeling for 60 min and α_1 PI was detected in cell lysates. M_r markers are indicated in the right margin.

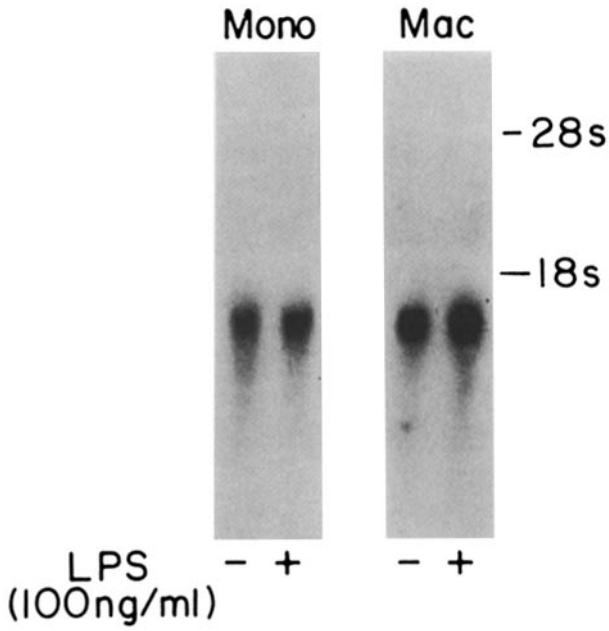

FIGURE 7. Effect of LPS on steady-state levels of α_1 PI mRNA in monocytes and macrophages. After 24 h in culture, monolayers of blood monocytes (*Mono*) or bronchoalveolar macrophages (*Mac*) were incubated for 12 h in serum-free medium alone (−) or supplemented with LPS, 100 ng/ml (+). RNA was then subjected to RNA blot analysis using radiolabeled α_1 PI cDNA as probe. Similar amounts of ethidium bromide–stained 18S and 28S were visualized in each lane. 18S and 28S ribosomal RNA is indicated in the right margin.

Pretranslational and Translational Mechanisms. Total cellular RNA was isolated from confluent monolayers of control or LPS-treated monocytes or macrophages and subjected to RNA blot analysis. In contrast to its effect on the rate of synthesis of α_1 PI, the effect of LPS on steady-state levels of α_1 PI mRNA is variable. In some experiments, accumulation of α_1 PI mRNA increases less than 2-fold in the presence of LPS (Fig. 7). In other experiments, steady-state levels of α_1 PI increase 2.5-fold (Fig. 8). The difference in the magnitude of the LPS effect on steady-state levels of α_1 PI mRNA (1.5–2.5-fold increase) and rate of

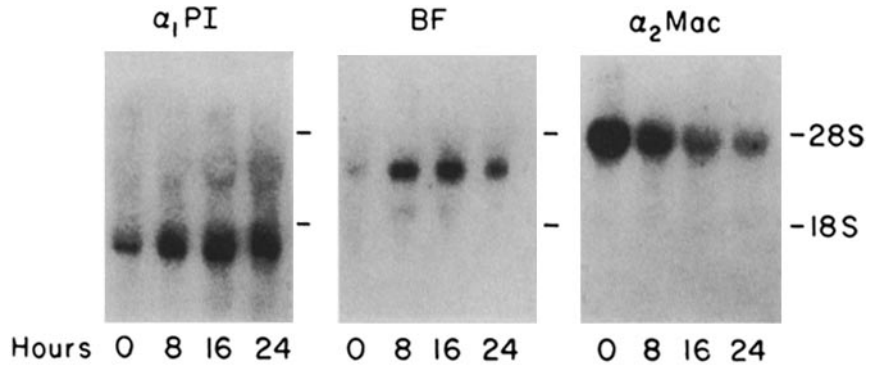

FIGURE 8. Time-dependent changes in α_1 PI mRNA in LPS-activated monocytes and macrophages. After 24 h in culture, monolayers of monocytes were incubated for 24 h in serum-free medium alone (0) or for the specified time intervals in serum-free medium supplemented with LPS, 100 ng/ml. RNA was then subjected to RNA blot analysis with radiolabeled α_1 PI, α_2 macroglobulin, or factor B cDNA as probe. Similar amounts of ethidium bromide–stained 18S and 28S RNA were visualized in each lane. 18S and 28S ribosomal RNA is indicated in the right margin.

synthesis of α_1 PI (4.5–8.7-fold increase) is not a result of time-dependent changes (Fig. 8) or dose-dependent changes (data not shown). LPS did mediate a 4–6-fold increase in steady-state levels of factor B mRNA and a decrease in α_2 macroglobulin mRNA levels in the same experiments (Fig. 8). These results suggest that the effect of LPS on expression of α_1 PI involves both pretranslational and translational mechanisms, but predominantly results from an increase in the efficiency of translation of α_1 PI mRNA. Although less likely, it is not possible to completely exclude the possibility that LPS decreases the rate of degradation of α_1 PI.

The Effect of LPS on Expression of α_1 PI in Monocytes From Deficient Individuals Exaggerates the Intrinsic Cellular Defect. The cellular defect in homozygous PiZZ α_1 PI deficiency, a selective decrease in the rate of secretion of α_1 PI, is expressed in monocytes from PiZZ individuals (2). Therefore, it is possible to examine the effect of enhanced translation of α_1 PI, as mediated by LPS, on a cell in which there is a defect in posttranslational processing/transport. Monocytes from PiMM and PiZZ individuals were separately incubated in control medium or medium supplemented with LPS and then subjected to pulse-chase radiolabeling (Fig. 9). The rate of synthesis of α_1 PI increases to a similar extent in PiMM and PiZZ monocytes (compare IC time 0 left top with right top and IC time 0 left bottom to IC time 0 right bottom). Although α_1 PI is already disappearing from the intracellular contents and appearing in the extracellular fluid by 30 min of the chase period in PiMM monocytes, it accumulates in intracellular contents over the entire chase period in PiZZ monocytes. These results indicate that upregulation of the expression of α_1 PI by a pretranslational mechanism, as mediated by a product of $T4^+$ lymphocytes (27) or by serine elastase (28), or by a translational mechanism, as mediated by LPS, results in a greater intracellular accumulation of α_1 PI in PiZZ monocytes.

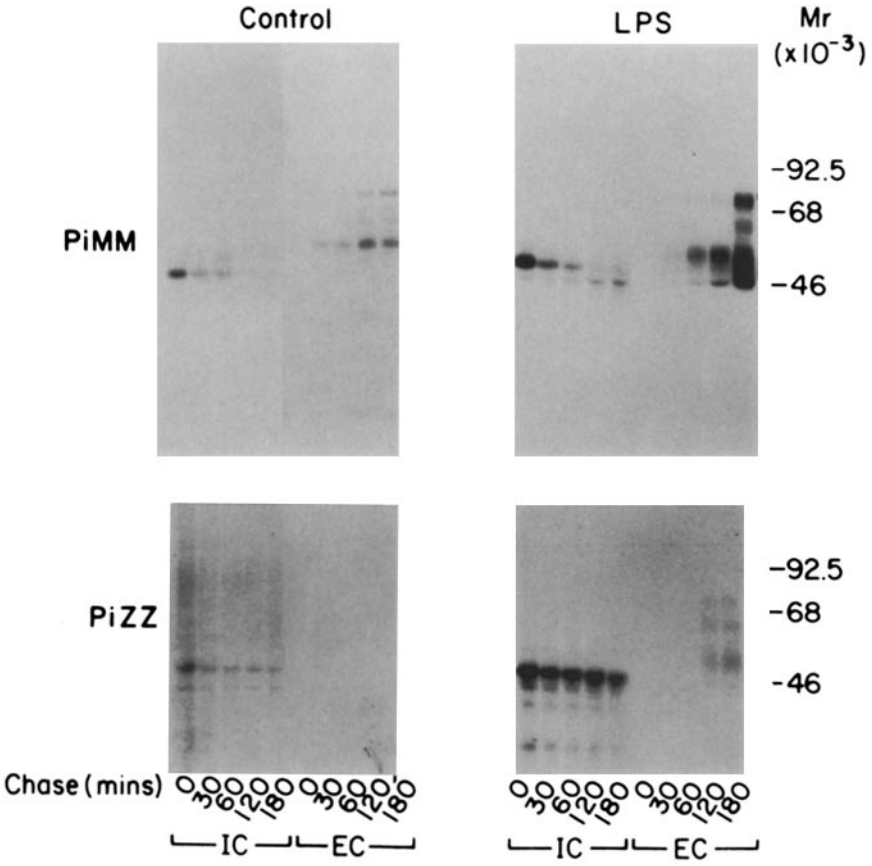

FIGURE 9. Effect of LPS on the kinetics of secretion of α_1 PI in PiMM and PiZZ monocytes. After 24 h in culture monocytes from PiMM (*top panels*) and PiZZ individuals (*bottom panels*) were incubated for 24 h in medium alone (control) or medium supplemented with LPS 100 ng/ml (LPS). Monolayers were then subjected to pulse-chase radiolabeling. M_r markers are indicated.

Discussion

These experiments demonstrate tissue-specific, predominantly translational regulation of the expression of α_1 PI in human mononuclear phagocytes by LPS. The increase in α_1 PI expression in blood monocytes and bronchoalveolar macrophages is part of a program of specific changes in expression of serine protease inhibitors that accompany activation by LPS. There is an increase in expression of C1 inhibitor and a decrease in expression of α_2 macroglobulin.

Changes in expression of α_1 PI and other serine protease inhibitors accompany many other specific changes in gene expression in macrophages during activation by LPS. Production of tumoricidal activity (47), superoxide anions (44), monokines (48, 49), prostaglandin metabolites (50), neutral proteases (51), proteases of the complement (40, 52, 53), coagulation (54), and fibrinolytic pathways (55), and proto-oncogenes (56) in human mononuclear phagocytes are regulated during LPS activation. Recent studies (49, 57, 58) of endotoxin-resistant states have suggested that regulation of macrophage gene expression by LPS involves

distinct transcriptional and translational control mechanisms. In cord blood monocytes, endotoxin mediates an increase in steady-state levels of C3 and factor B mRNA (58), but there is no accompanying change in the synthesis or secretion of C3 or factor B (57). In macrophages of the endotoxin-resistant C3H/HeJ mouse or in dexamethasone-treated macrophages of the endotoxin-sensitive C3H/HeN mouse there are increases in cachectin/TNF mRNA levels in response to endotoxin but no changes in the accumulation of cachectin in the extracellular fluid (49). A conserved sequence of 33 nucleotides composed entirely of A and T residues has been noted in the 3′-untranslated region of genes encoding several cytokines that are regulated by LPS, including cachectin/TNF, lymphotoxin, IL-1, granulocyte/macrophage colony-stimulating factor, and the IFNs (59). This sequence is thought to be a recognition signal for degradation of specific mRNAs (60) and to be involved in posttranscriptional stabilization of these mRNAs in response to LPS (61). This sequence is not found in the 3′-untranslated region of the human α_1 PI gene (62). Moreover, there is a variable and less significant increase in steady-state levels of α_1 PI mRNA levels in response to endotoxin, indicating that a predominantly translational mechanism is involved in this particular regulatory effect of endotoxin.

The results of these experiments also extend our understanding of the factors that affect the net biological activity of macrophage-derived α_1 PI in tissues. During homeostasis, α_1 PI activity may be affected by the state of differentiation of the mononuclear phagocyte, by the local microenvironment, by interaction with serine elastase (1), by interaction with metallo elastases (46), as well as with cysteine proteases (45). During inflammation or tissue injury, expression of α_1 PI in cells of mononuclear phagocyte lineage may be directly regulated by products of activated lymphocytes (27), by proteolytic enzymes such as elastase (28), by products of bacteria such as LPS, and probably by products of oxidative metabolism (63). In contrast, expression of α_1 PI in the only well-characterized human liver cell culture systems, human hepatoma cell lines HepG2 and Hep3B, is regulated to only a limited extent. The rate of synthesis of α_1 PI may be increased by twofold in HepG2 cells incubated with crude supernatants of alloantigen-stimulated $T4^+$ clones (27, 28), but is not affected by IL-1, TNF (25), IFN-γ or crude supernatants of LPS-stimulated peripheral blood mononuclear cells (26). Regulation of other hepatic acute-phase genes in these cell lines is mediated by each of these cytokines or cytokine preparations (25, 26, 64). These observations raise the possibility that in humans, expression of α_1 PI by hepatocytes is largely constitutive while that of macrophages is regulated.

Expression of α_1 PI in macrophages from PiZZ individuals is also regulated by LPS. In this instance, an increase in the efficiency of translation of α_1 PI mRNA may further compromise the deficient host. Rate of synthesis of α_1 PI increases but rate of secretion is not affected. The result is greater intracellular accumulation of the inhibitor. An increase in steady-state levels of α_1 PI mRNA as mediated by crude lymphokine supernatants (27) or by serine elastase (28) also results in greater intracellular accumulation of α_1 PI in deficient macrophages. Nevertheless, the effect of LPS in the deficient individual may be especially profound since it also directly downregulates the expression of α_2 macroglobulin,

another elastase inhibitor expressed in macrophages at local tissue sites of inflammation or tissue injury.

Summary

α_1 Proteinase inhibitor (PI) is the principle inhibitor of neutrophil elastase, an enzyme that degrades many components of the extracellular matrix. Expression and regulation of α_1 PI, therefore, affects the delicate balance of elastase and antielastase, which is critical to turnover of connective tissue during homeostasis, tissue injury, and repair. In this study we show that expression of α_1 PI in human monocytes and macrophages is regulated during activation by LPS. LPS mediates a concentration- and time-dependent increase in the rate of synthesis of α_1 PI in mononuclear phagocytes. There is a 4.5–8.7-fold increase in functionally active inhibitor delivered to the cell culture fluid of monocytes. The effect of LPS is specific in that it is neutralized by an mAb to the lipid A moiety. The increase in expression of α_1 PI mediated by LPS occurs in the context of other specific changes in the expression of serine proteinase inhibitor genes in mononuclear phagocytes. There is an increase in the rate of synthesis of C1 inhibitor and a decrease in synthesis of α_2 macroglobulin. Regulation of α_1 PI by LPS is distinctive in that it is largely determined by a change in the efficiency of translation of α_1 PI mRNA. LPS has no effect on the rate of posttranslational processing and/or secretion of α_1 PI and, therein, causes greater intracellular accumulation of α_1 PI in mononuclear phagocytes from individuals with homozygous PiZZ α_1 PI deficiency.

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