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*J Immunol* 2006; 177:5550-5557; doi: 10.4049/jimmunol.177.8.5550

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Involvement of the Urokinase Kringle Domain in Lipopolysaccharide-Induced Acute Lung Injury

Xue-Qing Wang,* Khalil Bdeir,† Sergei Yarovoi,† Douglas B. Cines,† Wenfeng Fang,*§ and Edward Abraham‡‡

Urokinase plasminogen activator (uPA) plays a major role in fibrinolytic processes and also can potentiate LPS-induced neutrophil activation through interactions with its kringle domain (KD). To investigate the role of the uPA KD in modulating acute inflammatory processes in vivo, we cloned and then developed Abs to the murine uPA KD. Increased pulmonary expression of uPA and the uPA KD was present in the lungs after LPS exposure. Administration of anti-kringle Abs diminished LPS-induced up-regulation of uPA and uPA KD in the lungs, and also decreased the severity of LPS-induced acute lung injury, as determined by development of lung edema, pulmonary neutrophil accumulation, histology, and lung IL-6, MIP-2, and TNF-α cytokine levels. These proinflammatory effects of the uPA KD appeared to be mediated through activation of Akt and NF-κB. The present studies indicate that the uPA KD plays a major role in the development of TLR4-mediated acute inflammatory processes, including lung injury. Blockade of the uPA KD may prevent the development or ameliorate the severity of acute lung injury induced through TLR4-dependent mechanisms, such as would occur in the setting of Gram-negative pulmonary or systemic infection. The Journal of Immunology, 2006, 177: 5550–5557.

Urokinase plasminogen activator (uPA) is a serine protease that plays a major role in fibrinolytic processes, where it facilitates the conversion of plasminogen to plasmin (1–3). In addition to its role in modulating coagulation, there are recent data showing that uPA has potent proinflammatory properties. In particular, exposure of LPS-stimulated neutrophils to uPA produces enhanced nuclear translocation of the transcriptional regulatory factor NF-κB, activation of the kinase Akt, and release of cytokines, such as TNF-α (4). The ability of uPA to potentiate neutrophil activation appears to have in vivo significance, because transgenic mice unable to produce uPA are protected from acute inflammatory processes in which neutrophils play a major role, such as LPS-induced acute lung injury (4). Because circulating and pulmonary levels of uPA are elevated for prolonged periods in patients with severe infections, including pneumonia (5, 6), these findings suggested that blockade of these nonfibrinolytic functions of uPA might have therapeutic use in such pathophysiologic settings.

The domains in the uPA molecule that are responsible for its proinflammatory effects and the receptors involved in uPA-induced neutrophil activation are distinct from those responsible for its fibrinolytic or chemotactic properties (7, 8). In particular, while the single-chain proenzyme form of uPA possesses little or no proteolytic activity, it is still able to potentiate LPS-induced neutrophil activation (4). uPA is composed of three structurally independent components: a growth factor domain (GFD; aa 1–46), a kringle domain (KD; aa 47–135), and a proteolytic domain (PD; aa 159–411). The GFD is responsible for the interaction of urokinase with the uPAR/CD87 receptor (2, 9, 10) and participates in uPA-dependent neutrophil chemotaxis, while the PD includes the catalytically active site of the enzyme. Recent experiments from our laboratory demonstrated that the KD is responsible for the ability of uPA to enhance LPS-induced neutrophil activation through a uPAR/CD87-independent mechanism that involves αβ integrins (11). In particular, whereas addition of purified KD to LPS-stimulated neutrophils increased cytokine release, this effect was lost when deletion mutants of uPA lacking the KD were used in the place of the KD or intact single-chain uPA (scuPA).

Although our previous results indicated that the uPA KD can potentiate activation of NF-κB and cytokine production by LPS-stimulated neutrophils, those experiments only used isolated neutrophils and the in vivo role of the KD in modulating inflammatory processes remained unknown. We examined this issue in the present studies by determining whether treatment with specific anti-mouse KD Abs affected the severity of LPS-induced acute lung injury. Acute lung injury is a frequent and life-threatening clinical problem, affecting over 200,000 patients per year in the United States alone, and associated with mortality rates of >25% as well as substantial morbidity (12, 13). These experiments demonstrate not only that the uPA KD is up-regulated in acutely inflamed lungs, but also that blockade of the KD diminishes the severity of this inflammatory process.

Materials and Methods

Mice

Male BALB/c mice, 6–8 wk of age, were purchased from Harlan Sprague Dawley. The mice were kept on a 12-h light/dark cycle with free access to...
food and water. All experiments were conducted in accordance with institutional review board-approved protocols.

Materials

Isolofurane was obtained from Abbott Laboratories. Escherichia coli 0111:B4 endotoxin was purchased from Sigma-Aldrich. RPMI 1640/25 mM HEPES/glutamine was obtained from Mediatech, while FBS and penicillin/streptomycin were purchased from Mediatech. Custom mixture Abs and columns for neutrophil isolation were purchased from Stem Cell Technologies. Abs to scuPA, uPA KD, as well as all other solutions, measured by ELISA (BioWhittaker), was <1 pg/ml.

Cloning of murine uPA KD and preparation of anti-KD Abs

Mouse cDNA encoding the KD of uPA (aa 48–144) (Fig. 1A) was amplified by PCR, then subcloned into BglII and AgeI cloning sites of the plasmid pMT/BIP/V5-HisA (Invitrogen Life Technologies). S2 Drosophila cells (Invitrogen Life Technologies) were transfected with the construct. Stable transfectants were selected and used to express mouse uPA kringle in serum-free medium as recommended by the supplier. The secreted protein was purified from the medium on a SP Trisacryl M column (Sigma-Aldrich), eluted with high salt buffer, filtered twice through an Amicon Ultra Centrifugal Filter (30,000 Da molecular mass cutoff), and buffer was exchanged to PBS using an Amicon Ultra Centrifugal Filter (5,000 Da molecular mass cutoff). The final protein appears as a single band that migrates at expected size on 10% Bis-Tris SDS-PAGE (Fig. 1B). Goat uPA kringle Abs were obtained by immunization with recombinant murine kringle, with boosting every 2 wk, and final harvest of serum after 8 wk, followed by purification of total IgG (Rockland Immunochemicals).

Isolation of neutrophils

Mouse neutrophils were purified from bone marrow cell suspensions as previously described (4, 17, 18). Briefly, to obtain the bone marrow cell suspension, the femur and tibia of a mouse were flushed with 5 ml of RPMI 1640/penicillin/streptomycin and the cells passed through a glass wool column, and pelleted by centrifugation at 1000 rpm for 10 min. The cell pellets were resuspended in 0.3% FCS/PBS and then incubated with 20 μl of primary Abs specific for cell surface markers F4/80, CD4, CD45R, CD5, and TER119 for 15 min, rotating at 4°C. The entire cell suspension was then added, and the cells incubated for 15 min, rotating at 4°C. Following this, 60 μl of colloidal magnetic dextran iron particles was added to the suspension and incubated for 15 min, rotating at 4°C. The entire cell suspension was then placed into a column, surrounded by a magnet. The T cells, B cells, RBC, monocytes and macrophages. Anti-biotin tetrameric Ab complexes (100 μl) were then added, and the cells incubated for 15 min, rotating at 4°C. Following this, 60 μl of colloidal magnetic dextran iron particles was added to the suspension and incubated for 15 min, rotating at 4°C. The entire cell suspension was then placed into a column, surrounded by a magnet. The T cells, B cells, RBC, monocytes and macrophages were captured in the column, allowing the neutrophils to pass through by negative selection methods. After negative selection of neutrophils, using the Ab mixture, the cells were pelleted and washed several times. Because of the differential density of platelets, there was no contamination with platelets in the final isolated neutrophil population. Neutrophil purity, as determined by Wright’s stained cytopsin preparations, was >97%. Cell viability, as determined by trypan blue exclusion, was consistently >98%.

Culture of MH-s cells

The MH-S cell line was purchased from American Type Culture Collection and was derived by SV40 transformation of an adherent cell-enriched population of mouse alveolar macrophages. Cells were cultured in RPMI 1640 plus 2 mM t-glutamine and 10% FBS, at 37°C in 5% CO₂.

In vivo acute lung injury model

Acute lung injury was induced by intratracheal administration of 1 mg/kg O4:B111 E. coli LPS into BALB/c mice. With this model, acute lung injury, as characterized by neutrophil infiltration into the lung interstitium, development of interstitial edema, and increased proinflammatory cytokine production occurs after injection of LPS with the greatest accumulation of neutrophils into the airways and histologic injury being present 24 h after LPS exposure (19–21). For this procedure, a blunted-end 20-gauge needle was passed through the mouth into the trachea in an isoflurane-anesthetized mouse and the LPS solution (in 50 μl of sterile saline) was deposited. There were no deaths associated with this model for intratracheal LPS exposure.

Harvest of lungs and bronchoalveolar lavage

Lungs were perfused with iced saline and harvested 24 h after LPS administration. Bronchoalveolar lavages were obtained by cannulating the trachea with a blunt 20-gauge needle and then lavaging the lungs three times with 1 ml of iced PBS. The use of isoflurane anesthesia in control mice, not exposed to LPS, was not associated with any pulmonary histologic changes, as compared with past results with mice subjected to methoxyflurane or ether anesthesia (22–25).

Preparation of lung homogenates for ELISA and Western blot analysis

Lung tissues were homogenized as previously described (3). In brief, lung samples were homogenized in ice-cold lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 10 mM NaF, 300 μM p-nitrophenyl phosphate, 1 mM PMSF, 10 μg/ml leupeptin, and 10 mM HEPES/glutamine) as shown on Coomassie blue-stained molecular mass markers (lane M). The recombinant muPA-KD has a molecular mass of 5.2 kDa with the lowest molecular mass marker being at 5 kDa.

FIGURE 1. A, The recombinant murine uPA KD (muPA-KD), amino acids S48 to Q144 of murine mature uPA sequence, has two extra amino acids (RS) at the N terminus. B, Murine uPA kringle before filtering (lane 1), after single (lane 2), and after double passage through a 30,000 Da molecular mass cutoff filter (lane 2) as shown on Coomassie blue-stained SDS-PAGE gel with molecular mass markers (lane M). The recombinant muPA-KD has a molecular mass of 5.2 kDa with the lowest molecular mass marker being at 5 kDa.

FIGURE 2. A, Localization of uPA and uPA KD in tissues of unmanipulated mice. Western blots of tissue homogenates from designated sites were stained with anti-uPA or goat anti-mouse uPA KD (Anti-KD) Abs. Both Abs detected a 33-kDa band in lung tissue. B, Immunoprecipitation of uPA from lung homogenates and neutrophil lysates, followed by Western blotting with Abs to the uPA KD. Total cell lysates from lungs and neutrophils were used as positive controls.
pernatants were collected and assayed for TNF-α according to manufacturer's protocol (Pierce). Assayed using the micro BCA protein assay kit standardized to BSA, anti-uPA KD Abs (LPS goat IgG (LPS Aldrich). Homogenates were centrifuged at 14,000 g.

Lungs from either unmanipulated mice (A) or mice given LPS intratracheally. Twenty-four h previously (C and D) were stained with control goat IgG (A and C) or goat anti-uPA KD Abs (anti-KD) (B and D). Mice given LPS intratracheally were treated with control goat IgG (C) or goat anti-uPA kringle IgG (D) i.v. 10 min after LPS administration. Representative experiments at ×400 magnification are shown. Three additional experiments provided similar results.

Myeloperoxidase (MPO) assay

MPO activity was assayed as reported previously with minor modifications (23). In brief, lung tissue was homogenized in 1.0 ml of 50 mM potassium phosphate buffer (pH 6.0) containing a reducing agent, N-ethylmaleimide (10 mM) for 30 s on ice. The homogenate was centrifuged at 12,000 g for 30 min at 4°C. The pellet was homogenized once more in ice-cold buffer, and the homogenate was centrifuged. The pellet was resuspended and sonicated on ice for 90 s in 10× volume of hexadecyltrimethylammonium bromide (HTAB) buffer (0.5% HTAB in 50 mM potassium phosphate (pH 6.0)). Samples were incubated in a water bath (50°C) for 2 h and then centrifuged at 12,000 × g for 10 min. The supernatant was collected for assay of MPO activity as determined by measuring the H2O2-dependent oxidation of 3,3′-dimethoxybenzidine dihydrochloride at 460 nm. For determinations of MPO in bronchoalveolar lavage samples, specimens were obtained from LPS-treated or control mice after cannulation of the trachea and lavage with 1 ml of 4°C PBS, repeated three times. The MPO assay was performed using 20 μl of the bronchoalveolar lavage fluid, as described above.

Wet-to-dry lung weight ratios

The wet-to-dry ratio was determined as reported previously (26). All mice used for lung wet-to-dry weight ratios were of identical ages. Lungs were excised, rinsed briefly in PBS, blotted, and then weighed to obtain the “wet” weight. Lungs were then dried in an oven at 80°C for 7 days to obtain the “dry” weight.

Histopathology

Mouse lungs were inflation fixed with 10% formaldehyde at 20-cm H2O pressure and embedded in paraffin for sectioning at 6-μM thickness. The sections were stained with H&E.

Cytokine ELISA and protein assays

Immunoreactive TNF-α, IL-6, and MIP-2 were quantified using commercially available ELISA kits (R&D Systems), according to manufacturer’s instructions available ELISA kits (R&D Systems), according to manufacturer’s instructions.

**FIGURE 3.** Abs to uPA KD prevent kringle-induced enhancement of cytokine production by the murine alveolar macrophage cell line MH-S stimulated with LPS. MH-S cells were cultured without stimulation (Control) or with LPS alone (LPS), with 100 nM uPA KD and 3 μg/ml control goat IgG (LPS + KD + IgG) or with 100 nM uPA KD and 3 μg/ml goat anti-uPA KD Abs (LPS + KD + anti-KD). After 4 h of culture, the supernatants were collected and assayed for TNF-α or IL-6 protein. Use of 0.3 μg/ml anti-uPA KD Abs had no effect on LPS-induced cytokine production, *p < 0.05 vs control and #, p < 0.05 vs LPS or LPS + KD + anti-KD. Each condition was performed in five replicate wells. Results from a representative experiment are shown. Three additional experiments provided similar results.

**FIGURE 4.** Increased expression of the uPA KD in LPS-treated lungs. Lungs from either unmanipulated mice (A and B) or mice given LPS intratracheally. Twenty-four h previously (C and D) were stained with control goat IgG (A and C) or goat anti-uPA KD Abs (anti-KD) (B and D). Mice given LPS intratracheally were treated with control goat IgG (C) or goat anti-uPA kringle IgG (D) i.v. 10 min after LPS administration. Representative experiments at ×400 magnification are shown. Three additional experiments provided similar results.

**FIGURE 5.** A, uPA expression is increased in the lungs during the 24 h after LPS administration. B, Treatment with anti-uPA KD Abs (Anti-KD), but not control goat IgG (IgG), reduces pulmonary expression of uPA after LPS treatment. Lung homogenates were obtained in unmanipulated mice as well as 6 and 24 h after LPS administration in A. In B, mice were given either 50 μl of normal saline or 20 μg of LPS in 50 μl of normal saline intratracheally and 10 min later were treated i.v. with either goat IgG or goat anti-uPA KD IgG (Anti-KD). Lung homogenates were obtained 24 h after saline or LPS administration for Western blotting. Total ERK staining is shown as a control for protein application to the Western blots. Representative experiments are shown. Densitometry results from three independent experiments are presented. ***, p < 0.01 vs IgG treatment.**
instructions and as described previously (27). Total protein in bronchoalveolar lavage samples was measured using a protein assay kit (Bio-Rad), following the manufacturer’s instructions.

**EMSA**

Nuclear extracts were prepared and assayed by EMSA as previously described (27). For analysis of NF-κB, the κB DNA sequence of the Ig gene was used. Synthetic double-stranded sequences (with enhancer motifs underlined) were filled in and labeled with [α-32P]dATP using Sequenase DNA polymerase as follows: κB sequence, 5'-TTTTCGAGCTCGG GACCTTTCCGAGC-3' and 3'-GCTTCACGTCCTGAAAGCCTAGTTT-5'.

**Western blot analysis**

Western blots to detect levels of phosphorylated and total p38, Akt, ERK, and JNK were performed essentially as previously described (27, 28). Neutrophils were lysed in ice-cold lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na2 EDTA, 1% Triton X-100, 1 mM EGTA, 1 mM trisodium vanadate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM PMSE, 1 μg/ml leupeptin (pH 7.5), then resuspended and sonicated for 30 s. Debris from the lysed cell was pelleted by centrifugation at 14,000 rpm for 20 min. The supernatant was then removed and stored at −86°C. The protein concentration of each sample was assayed using the BCA protein assay kit (Pierce) standardized to BSA, according to manufacturer’s protocol. For the Western blots, 50 μg of protein was loaded and run on a 10% Tris-HCl SDS polyacrylamide gel. Protein was electrotransferred to a nitrocellulose membrane and then blocked with 5% nonfat dry milk, 20% Tris-HCl SDS polyacrylamide gel. The membranes were then stripped using stripping buffer (63 mM Tris-HCl (pH 6.8), 2% SDS, 100 mM 2-ME from Bio-Rad), and reprobed with Abs specific for total Akt, JNK, ERK, or p38 using a dilution of 1/3000 in 5% nonfat dry milk. After washing five times, bands were detected using ECL Western blotting detection reagents (Amersham Biosciences). The membranes were then stripped using stripping buffer (63 mM Tris-HCl (pH 6.8), 2% SDS, 100 mM 2-ME from Bio-Rad), and reprobed with Abs specific for total Akt, JNK, ERK, or p38.

**Statistical analysis**

For each experimental condition, the entire group of animals was prepared and studied at the same time. Data are presented as mean ± SD for each experimental group. One-way ANOVA, the Tukey-Kramer Multiple Comparisons test (for multiple groups), or Student’s t test (for comparisons between two groups) were used. A value of p < 0.05 was considered significant.

**Results**

**Preparation of Abs to the urokinase KD**

Titers of anti-uPA KD in postimmunization IgG reached 1/6400 compared with those present in preimmunization serum. The anti-KD Abs reacted with human scuPA, at dilutions as low as 1/6400, but showed no enhanced association, compared with preimmune goat IgG, with mutant human scuPA lacking the KD, at even dilutions of 1/200. To determine that the anti-uPA kringle IgG reacted specifically with mouse uPA, we screened mouse tissues for uPA with both anti-kringle IgG as well as anti-uPA Abs. As shown in Fig. 2A, the anti-kringle IgG bound to a 33-kDa protein in tissues, with the highest level of expression being in lung. Two additional anti-uPA Abs (Santa Cruz Biotechnology) also detected the same 33-kDa band on the Western blots. Further confirmation that the goat anti-kringle IgG binds directly to uPA in vivo was obtained from studies in which the anti-kringle IgG identified large amounts of uPA in lung homogenates immunoprecipitated with anti-uPA Abs (Fig. 2B). The anti-KD Abs also demonstrated activity against the proinflammatory effects of uPA in vitro, as shown by reduced production of TNF-α and IL-6 by LPS stimulated MH-s mouse macrophages cocultured with purified uPA KD (Fig. 3). In contrast, normal goat IgG control Abs had no effect on cytokine release.

**LPS treatment increases uPA kringle expression in the lungs**

Pulmonary expression of the uPA KD was increased 24 h after LPS exposure (Fig. 4). Under these conditions, the epithelium was the primary cell population staining for KD. Administration of anti-KD Abs immediately after intratracheal LPS injection resulted in diminished staining of the lungs for the uPA KD (Fig. 4D), suggesting that LPS-induced pulmonary production of uPA is likely to enhance subsequent generation of uPA in an autocrine
fashion and that blockade of the uPA KD, even if accomplished after pulmonary exposure to LPS, can reduce expression of uPA and the uPA KD at later time points, such as 24 h after LPS administration, as was examined in these experiments.

To exclude residual activity from injected anti-KD Abs, Western blotting was used to determine total uPA expression in the lungs. As shown in Fig. 5A, administration of intratracheal LPS resulted in increased amounts of immunoreactive uPA in the lungs, starting within 6 h, and pulmonary uPA concentrations were further increased by 24 h post-LPS exposure. In a similar manner to the immunohistochemistry results shown in Fig. 4, pulmonary uPA levels, as determined by Western blotting, were decreased when anti-uPA KD Abs were administered after the injection of LPS (Fig. 5B).

**Blockade of urokinase KD diminishes the severity of LPS-induced lung injury**

In previous studies using uPA−/− transgenic mice, we found that uPA played a central role in the development of LPS-induced acute lung injury (4). Because in vitro experiments demonstrated that the uPA KD was the proinflammatory component of uPA, responsible for the ability of uPA to enhance proinflammatory cytokine production by LPS-stimulated neutrophils (11), it seemed likely that the uPA KD might also participate in enhancing the severity of acute lung injury after LPS exposure, a pathophysiological process in which neutrophils play a central role (26). To examine this issue, mice were given LPS intratracheally and then treated with anti-KD Abs or control goat IgG. After 24 h, the severity of lung injury, as well as release of proinflammatory cytokines into the lungs, was determined.

As shown in Fig. 6, treatment with anti-KD Abs significantly reduced the severity of pulmonary edema, accumulation of neutrophils in the lung interstitium and in bronchoalveolar lavage fluid, as well as the increase in protein in bronchoalveolar lavage fluid produced by LPS exposure. Histological studies showed that there was less pulmonary injury, including diminished interstitial edema, leakage of red cells into the alveolar space, and accumulation of neutrophils into the pulmonary interstitium in mice given anti-KD Abs after LPS administration as compared with the control IgG-treated animals (Fig. 7). Anti-KD Abs also prevented LPS-induced increases in lung concentrations of TNF-α, MIP-2, and IL-6 (Fig. 8).

**FIGURE 7.** LPS-induced alterations in pulmonary histology are diminished by blockade of uPA KD. Mice were treated i.v. with either goat anti-uPA KD (anti-KD) Abs (A) or control goat IgG (B) 10 min after intratracheal LPS administration and then lungs were harvested for H&E staining 24 h after LPS exposure. Histology from an unmanipulated control mouse is shown in C. Representative images at 200 magnification are shown. Two additional experiments provided similar results.

**FIGURE 8.** Blockade of uPA KD inhibits LPS-induced cytokine production in the lungs (TNF-α in A, MIP-2 in B, and IL-6 in C). Mice were treated i.v. with normal saline (saline), control goat IgG, or goat anti-uPA KD Abs (anti-KD) 10 min after intratracheal administration of saline or LPS and then lungs homogenates were harvested for cytokine determination 24 h after LPS exposure. Results are shown as picograms of cytokine per milligram of total lung protein. Groups of six mice were used for each condition. *p < 0.05 vs saline; #, p < 0.05 vs LPS saline and LPS IgG.

**Administration of Abs to the urokinase KD decreases LPS-induced NF-κB and Akt activation in the lungs**

Intratracheal administration of LPS resulted in increased nuclear translocation of NF-κB and activation of the kinases p38, Akt,
JNK, and ERK1/2 in lung homogenates, findings similar to those previously reported by ourselves and others (27, 29, 30). Treatment with anti-KD Abs, but not preimmune control goat IgG, was associated with decreased nuclear concentrations of NF-κB in lung homogenates and also diminished LPS-induced phosphorylation of Akt (Fig. 9), but had no effect on phosphorylation of p38, JNK, or ERK1/2 (data not shown), indicating that the enhanced expression of the uPA KD was responsible for the enhanced activation of NF-κB and Akt in LPS-exposed lungs, but not that of the other kinases examined.

Discussion

The present experiments demonstrate that the uPA KD is involved in modulating the severity of LPS-induced acute lung injury. In these studies, immunoreactive uPA and uPA KD were increased in the lungs for >24 h after LPS administration. The prolonged presence of uPA in the lungs is consistent with our previous results where elevated levels of uPA were found in bronchoalveolar lavage samples from humans given intrabronchial LPS 16 h previously (31). Persistent increases in pulmonary concentrations of uPA have also been found in patients with pneumonia (5, 6). However, in both animals and humans exposed to pulmonary LPS, as well as in patients with pneumonia, pulmonary concentrations of plasminogen activator inhibitor 1 (PAI-1) are elevated to an even greater degree than are those of uPA (5, 6, 31–34). The relatively greater levels of PAI-1 as compared with uPA appear to be largely responsible for the inhibition of the fibrinolytic activity of uPA in these settings.

The uPA KD may directly activate neutrophils through receptors other than those classically used by uPA, such as the uPAR. In previous in vitro studies (11), we found that αvβ3 integrins, rather than uPAR, were involved in ability of the uPA KD to enhance LPS-induced proinflammatory cytokine production by neutrophils. In addition, interactions between PAI-1 and uPA may modify activation pathways involving uPA through prolonging cellular exposure to the uPA KD or altering uPA conformation (35, 36). Association of PAI-1 and uPA involves the PD of uPA and plasminogen activator-receptor-related protein/α2-macroglobulin receptor (7, 37). The high levels of immunoreactive uPA found in the lungs after LPS exposure or in the setting of pneumonia, although inactive in terms of fibrinolysis, could still have potent effects on the activation of neutrophils, and perhaps of other pulmonary cell populations, such as alveolar macrophages, that are involved in inflammatory responses. Of note, recent results from our laboratory indicated that not only does PAI-1 not inhibit the proinflammatory effects of uPA and the uPA KD on LPS-induced neutrophil activation, but it also actually has proinflammatory effects of its own that are additive with those of uPA and the uPA KD. It is also possible that the uPA KD modulates the development of acute lung injury through mechanisms that do not directly involve neutrophils, such as potentiating the release of proinflammatory mediators by alveolar macrophages or epithelial cells that are also activated through their exposure to LPS. Increased production of chemokines and other chemoattractant molecules by such pulmonary cell populations can contribute to neutrophil accumulation in the lungs and subsequent injury. Finally, it is possible that the uPA KD may directly enhance neutrophil chemotaxis, as has been shown for smooth muscle cells (38), thereby...
facilitating neutrophil migration into the pulmonary interstitium and air spaces.

The procoagulant environment which exists in the lungs during infection has been postulated to result in pulmonary injury primarily through alterations in the microvasculature, including those induced by the presence of thrombus formation, producing endothelial cell activation and initiation of acute inflammatory processes (39, 40). However, the present studies, coupled with our previous results (4) demonstrating that uPA potentiates LPS-induced neutrophil activation and contributes to lung injury, indicate that the prolonged presence of uPA, and particularly of the uPA KD, in infected and LPS-exposed lungs is likely to enhance injury not necessarily through involvement of the endothelium, but rather through interactions with neutrophils that accumulate in large numbers in the pulmonary parenchyma and air spaces in these conditions. Of note, our immunohistochemistry studies found that uPA KD expression was primarily localized to epithelial cells of LPS-exposed lungs. The presence of both uPA and LPS in the alveolar space, microvasculature, and other regions of the lungs during pulmonary infection is capable of enhancing neutrophil activation, including the release of proinflammatory cytokines, which then lead to potentiation of lung injury.

An important mechanism by which scuPA potentiates the production of proinflammatory cytokines by LPS-stimulated neutrophils under in vitro conditions is through increasing activation of the kinase Akt that then contributes to enhanced nuclear translocation of NF-κB (4). In the present in vivo studies, the uPA KD also appears to induce activation of Akt and nuclear translocation of NF-κB in the setting of pulmonary exposure to LPS. In particular, in the present experiments, blockade of the uPA KD resulted in diminished activation of Akt, but not of alternate kinases known to participate in LPS-induced acute lung injury, such as p38, ERK1/2, or JNK.

We and others (27, 41–43) have shown that PI3K, which are immediately upstream of Akt, are involved in LPS-induced neutrophil activation. In particular, the PI3Kγ isoform appears to play an important role in inducing nuclear translocation of NF-κB and production of proinflammatory cytokines by LP-stimulated neutrophils (27). PI3Kγ also participates in LPS-induced acute lung injury (27). Because of the immediately proximate position of PI3K, and particularly PI3Kγ, in Akt activation in neutrophils, it is possible that these phosphoinositide kinases are involved in the potentiation of neutrophil activation and lung injury by the uPA KD. We are actively investigating this possibility.

The present studies suggest that blockade of the uPA KD may prevent the development or ameliorate the severity of acute lung injury induced through TLR4-dependent mechanisms, such as would occur in the setting of Gram-negative pulmonary or systemic infection. Because of the similarity in signaling pathways downstream of TLR2 and TLR4, and particularly the activation of Akt that occurs after engagement of both TLR2 and TLR4, anti-KD therapies may also be beneficial in the setting of Gram-positive infections, although this remains to be proven directly. The uPA KD may be a particularly attractive target for inhibitory therapies in acute lung injury. Blockade of early proinflammatory events, such as the release of TNF-α or IL-1, was not found to be effective in clinical trials for sepsis, presumably because relatively few patients continued to have elevated levels of these mediators at the time of admission to the hospital and intensive care unit (44, 45). In contrast, as pulmonary concentrations of uPA remain elevated for prolonged periods after exposure to LPS, in the presence of pneumonia, and also in systemic infections with pulmonary involvement, it is possible that anti-KD Abs or other specific inhibitory therapies, such as nonactivating kringle-based peptides, may still be capable of diminishing the severity of acute lung injury while maintaining urokinase binding to its receptor and its contribution to the fibrinolytic balance. The beneficial effects of therapies aimed at blocking KD-mediated inflammatory effects may be effective even if administered after this pathophysiologic process first becomes clinically evident if the continued presence of KD in the lungs participates in ongoing inflammation and late-stage fibrotic processes. Future clinical trials will be necessary to address this hypothesis.

**Disclosures**

The authors have no financial conflict of interest.

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