Protective Role of Urinary Trypsin Inhibitor in Acute Lung Injury Induced by Lipopolysaccharide

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Urinary trypsin inhibitor (UTI), a serine protease inhibitor, has been widely used as a drug for patients with acute inflammatory disorders such as disseminated intravascular coagulation, shock, and pancreatitis. However, direct contribution of UTI to inflammatory diseases has not been established. The present study analyzed acute inflammatory lung injury induced by lipopolysaccharide (LPS) in UTI-deficient (–/–) mice and corresponding wild-type (WT) mice. UTI (–/–) and WT mice were treated intratracheally with vehicle or LPS (125 μg/kg). The cellular profile of bronchoalveolar lavage fluid, lung water content, histology, and expression of proinflammatory molecules in the lung were evaluated. After LPS challenge, both genotypes of mice revealed neutrophilic lung inflammation and pulmonary edema. UTI (–/–) mice, however, showed more prominent infiltration of inflammatory cells and edema than WT mice. After LPS challenge in both genotypes of mice, the lung levels of mRNA and/or protein expression of interleukin-1β, macrophage inflammatory protein-1α, macrophage chemoattractant protein-1, keratinocyte chemoattractant, and intercellular adhesion molecule-1 (ICAM-1) were elevated in both groups, but to a greater extent in UTI (–/–) mice than in WT mice. These results suggest that UTI protects against acute lung injury induced by bacterial endotoxin, at least partly, through the inhibition of the enhanced local expression of proinflammatory cytokines, chemokines, and ICAM-1. Exp Biol Med 230:281–287, 2005

Key words: urinary trypsin inhibitor; lipopolysaccharide; acute lung injury; cytokines; chemokines; intercellular adhesion molecule

Introduction

Acute respiratory distress syndrome (ARDS) is generally refractory to clinical control and a major cause of death in intensive care units. Furthermore, the recent outbreak of severe acute respiratory syndrome emphasizes the lethal outcomes associated with acute lung injury (1). Therefore, it is important to understand the pathogenesis of acute lung injury and to find beneficial strategies for its treatment. Exposure of the lower respiratory tract to lipopolysaccharide (LPS) is a well-known model of acute lung injury (2). LPS activates alveolar macrophages and causes neutrophils to infiltrate and damage the lungs (3). The stimulated neutrophils produce various molecules, such as platelet-activating factor, arachidonic acid metabolites, cytokines, free radicals, and proteases (4).

Among proteases, neutrophil elastase and cathepsin G have been shown to play an important role in LPS-induced shock, which was demonstrated in a previous study using knockout mice (5). In addition, another recent study using knockout mice has reported that a secretory leukoprotease inhibitor, one of the serine protease inhibitors, plays a protective role in LPS-induced shock (6). These studies have indicated that proteases are critical mediators in endotoxin-related injuries.

Urinary trypsin inhibitor (UTI) is a multivalent Kunitz-type serine protease inhibitor that is found in human urine and blood (7). UTI, also referred to as ulinastatin, human inhibitor of 30kDa, *Ascaris* chymotrypsin/elastase inhibitor-1, or bikunin, is an acidic glycoprotein with a molecular weight of 30 kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (7–9). It is composed of 143 amino acid residues, and its sequence includes two Kunitz-type domains (7). UTI has been widely used in Japan as a drug for patients with disseminated intravascular coagulation (DIC), shock, and pancreatitis. UTI mainly inhibits proteases including trypsin, α-chymotrypsin, plasmin,

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cathepsin G, and leukocyte elastase, as well as proteases in the coagulation cascade. UTI has been reported to have anti-inflammatory properties *in vitro* apart from blocking the protease pathway. UTI inhibits the enhanced production of proinflammatory molecules such as thromboxane B2 (TXB2) (10), interleukin-8 (IL-8) (11), tumor necrosis factor-α (TNF-α) (12) induced by LPS, and the production of prostaglandin H_2 synthase (PHS-2), which is induced in some inflammatory conditions and whose metabolites can contribute to the inflammatory process (13) *in vitro*. In addition, UTI ameliorates several inflammatory models such as ischemia-reperfusion injury (14), hemorrhagic shock (15), septic shock (16), and glomerulonephritis (17) *in vivo*. In these models, however, the animals have been treated with human-derived UTI as a foreign protein; thus, the direct contribution of UTI in inflammatory diseases including acute lung injury has never been examined in knockout mice.

In the current study, we explored the role of UTI in acute lung injury induced by intratracheal instillation with LPS using UTI (–/–) mice and wild-type (WT) mice. We also elucidated the role of UTI in the lung expression of proinflammatory molecules including cytokines, chemokines, and intercellular adhesion molecule-1 (ICAM-1).

Methods

Mice. The studies were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health. All animal studies were approved by the Institutional Review Board. The generation of mice deficient in UTI gene, which was confirmed by Western blotting using an anti-UTI antibody, and normal control littermates (C57BL/6) was described previously (7). These mice were bred and maintained under a 12:12-hr light:dark cycle in our Level B pathogen-free facility. Male mice of both genotypes were used at 10–12 weeks of age and 27–31 g in weight.

UTI (–/–) and WT mice were treated with vehicle or LPS (*Escherichia coli* B55:05, Difco Lab, Detroit, MI). In both genotypes, the vehicle groups intratracheally received 100 μl of phosphate-buffered saline at pH 7.4 (Nissui Pharmaceutical Co., Tokyo, Japan). The LPS groups received 125 μg/kg of LPS dissolved in 100 μl of the same vehicle. Intratracheal administration was conducted as described previously (18).

Histologic Evaluation. After exsanguination, the lungs were fixed by intratracheal instillation of 10% neutral phosphate-buffered formalin at a pressure of 20 cm H_2O for at least 72 hrs. Slices 2 to 3 mm thick of all pulmonary lobes were embedded in paraffin. Sections 3 μm thick were stained with hematoxylin-eosin. Neutrophil infiltration was assessed by averaging the number of neutrophils enumerated in 30 randomly selected, high power fields (HPFs; ×400) in each slide. Histologic sections were evaluated in a blind fashion.

Lung Water Content. Twenty-four hours after the intratracheal administration of LPS or vehicle, blood was drawn and the bilateral lungs removed and dried in an oven at 95°C for 48 hrs. Thereafter, lung water content was estimated by calculating the ratio of the wet lung weight to the dry lung weight (mg) per gram of body weight (19).

Bronchoalveolar Lavage (BAL). BAL and cell counts in BAL fluid were conducted as previously reported by Takano *et al.* (18). In brief, the trachea was cannulated after the collection of blood. The lungs were lavaged with 1.2 ml of sterile saline at 37°C, instilled bilaterally by syringe. The lavaged fluid was harvested by gentle aspiration. This procedure was conducted two more times. Average volume retrieved was more than 90% of the 3.6 ml that was instilled; the amounts did not differ among treatments. The fluid collections were combined and cooled to 4°C. The lavage fluid was centrifuged at 300 *g* for 10 mins, and the total cell count was determined on a fresh fluid specimen using a hemocytometer. Differential cell counts were assessed on cytologic preparations. Slides were prepared using an Autosmear (Sakura Seiki Co., Tokyo, Japan) and were stained with Diff-Quik (International Reagents Co., Kobe, Japan). A total of 500 cells were counted under oil immersion microscopy.

Measurement of IL-1β and Chemokines in Lung Tissue Supernatants. After the collection of blood, the lungs were subsequently homogenized with 10 m*M* potassium phosphate buffer (pH 7.4) containing 0.1 m*M* EDTA (Sigma, St. Louis, MO), 0.1 m*M* phenylmethanesulfonyl fluoride (Nacalai Tesque, Kyoto, Japan), 1 μ*M* pepstatin A (Peptide Institute, Osaka, Japan), and 2 μ*M* leupeptin (Peptide Institute) as described previously (18). The homogenates were then centrifuged at 105,000 *g* for 1 hr. The supernatants were stored at –80°C. Enzyme-linked immunosorbent assays (ELISAs) for IL-1β (Endogen, Cambridge, MA), macrophage inflammatory protein-1α (MIP-1α), macrophage chemoattractant protein-1 (MCP-1), and keratinocyte chemoattractant (KC) (R&D Systems, Minneapolis, MN) in the lung tissue supernatants were conducted using matching antibody pairs according to the manufacturer's instructions. The second antibodies were conjugated to horseradish peroxidase. Subtractive readings of 550 nm from the reading at 450 nm were converted to pg/ml using values obtained from standard curves generated with varying concentrations of recombinant IL-1β, MIP-1α, MCP-1, and KC, with limits of detection of 3 pg/ml, 1.5 pg/ml, 10 pg/ml, and 2 pg/ml, respectively.

Extraction of mRNA and Reverse Transcription–Polymerase Chain Reaction (RT-PCR) Analysis. Total RNAs in the lung were extracted with Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. mRNA was prepared using Oligotex-dT30 mRNA purification kits (Takara, Kyoto, Japan), and

Table 1. Lung Water Content and Total Cells and Neutrophils in Bronchoalveolar Lavage Fluid (BALF) in WT and UTI (–/–) Mice Challenged with Lipopolysaccharide (LPS: 125 μg/kg)[a]

Genotype	Challenge	Lung water content (lung wet weight–dry weight [mg]/body weight [g])	Total cells in BALF ($\times 10^4$)	Neutrophils in BALF ($\times 10^4$)
WT	Vehicle	4.01 ± 0.07	16.23 ± 1.59	1.70 ± 0.54
	LPS	5.54 ± 0.12*	453.57 ± 28.24**	393.62 ± 17.64**
UTI (–/–)	Vehicle	4.32 ± 0.09	23.58 ± 2.06	2.47 ± 1.52
	LPS	6.57 ± 0.76*#	565.36 ± 45.21**#	538.76 ± 43.69**##

[a] To quantitate pulmonary edema, the lung water content was measured 24 hrs after the intratracheal instillation. The ratio of the wet lung weight–dry lung weight (mg)/body weight (g) was calculated. Results are means plus or minus standard error of the mean ($n = 5$ in each group). In another experiment, 24 hrs after the intratracheal administration of vehicle or LPS, lungs were lavaged for the analysis of BALF. The total cell count was determined on a fresh fluid specimen using a hemocytometer. Differential cell counts were assessed on cytologic preparations stained with Diff-Quik. Results are means plus or minus standard error of the mean ($n = 10$).

* $P < 0.05$ versus vehicle-treated mice.

** $P < 0.01$ versus vehicle-treated mice.

# $P < 0.05$ versus LPS-treated WT mice.

$P < 0.01$ versus LPS-treated WT mice.

cDNA synthesis was conducted according to the manufacturer's protocol.

The quantitation of mRNA expression was carried out by real time RT-PCR using the ABI Prism 7000 sequence detection system (TaqMan, Perkin-Elmer Corp., Foster City, CA), according to the manufacturer's instructions. cDNAs were amplified according to the thermal profile of 50°C for 2 mins then 95°C for 10 mins, followed by up to 40 cycles at 95°C for 15 secs and 60°C for 1 min. Specific primers and probes were obtained from Applied Biosystems. The sequences of 18S rRNA, IL-1β, MIP-1α, MCP-1, KC, and ICAM-1, which were purchased from Perkin-Elmer, were not disclosed by the manufacturer. The relative intensity of gene expression was quantitated by the standard method according to the manufacturer's protocol and normalized to an endogenous control gene (18S rRNA).

Statistical Analysis. Data were reported as mean plus or minus standard error of the mean. Differences between groups in histologic evaluation and relative intensity of gene expression were analyzed by Kruskal-Wallis test followed by Mann-Whitney *U* test using Statview. Differences in other data were examined for statistical significance using one-way ANOVA with a *post hoc* analysis (Fisher's Protected Least Squares Differences test). Significance was assigned to *P* values smaller than 0.05.

Results

UTI Protects Against Acute Lung Injury Induced by Bacterial Endotoxin. To examine the role of UTI in pulmonary edema related to bacterial endotoxin, we evaluated the lung water content 24 hrs after the intratracheal instillation with vehicle or LPS (Table 1). LPS treatment increased lung water content in both genotypes of mice ($P < 0.05$) compared with vehicle treatment. UTI (–/–) mice, however, showed a significantly greater increase in the lung water content when compared with WT mice ($P < 0.05$) following LPS treatment.

To determine the role of UTI in neutrophilic lung inflammation induced by bacterial endotoxin, we investigated the cellular profile of BAL fluid 24 hrs after the intratracheal instillation. In both genotypes of mice, LPS treatment induced significant increases in the numbers of total cells and neutrophils as compared with vehicle treatment ($P < 0.01$; Table 1). LPS treatment caused greater and significant increases in the numbers of BAL total cells ($P < 0.05$) and neutrophils ($P < 0.01$) in UTI (–/–) mice than in WT mice.

To determine the differences in the histologic changes after LPS treatment in the presence or absence of UTI, we evaluated lung specimens stained with hematoxylin-eosin 24 hrs after the intratracheal instillation. In the presence of LPS, WT mice showed moderate infiltration of neutrophils (Fig. 1B). In UTI (–/–) mice, LPS treatment led to a marked recruitment of neutrophils and interstitial edema (Fig. 1D). Vehicle administration alone caused no histologic changes in either WT (Fig. 1A) or UTI (–/–) (Fig. 1C) mice.

We performed morphometric analysis to quantitate the number of neutrophils in the lung 24 hrs after LPS challenge. As compared with vehicle treatment, LPS treatment significantly increased the numbers of neutrophils in the lung ($P < 0.01$; Fig. 1E) from both genotypes of mice. In the presence of LPS, UTI (–/–) mice showed significantly increased numbers of neutrophils in the lung ($P < 0.01$; Fig. 1E) when compared with WT mice.

UTI Deficiency Enhances Protein Expression of Proinflammatory Cytokines and Chemokines Related to Bacterial Endotoxin. To investigate the role of UTI in the protein expression of proinflammatory cytokines and chemokines related to bacterial endotoxin, we compared the protein levels of IL-1β, MIP-1α, MCP-1, and KC in the lung tissue supernatants among the four

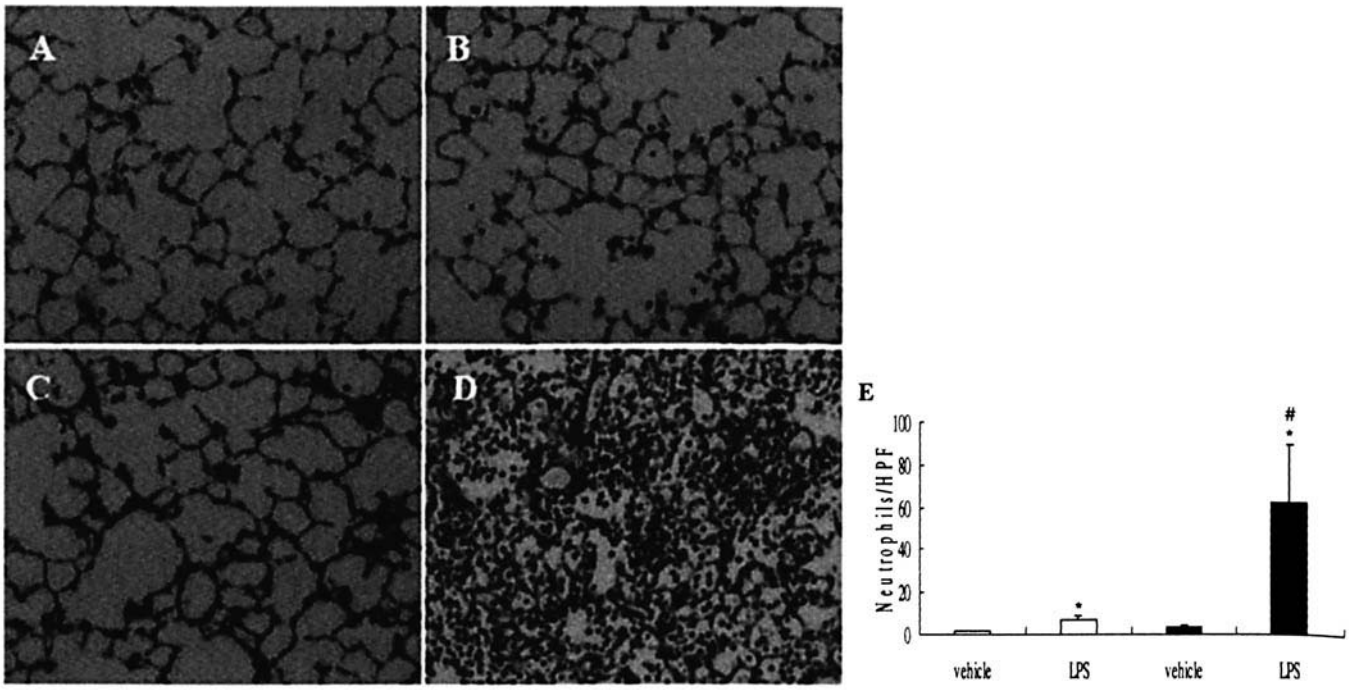

Figure 1. Histopathologic findings of the lung obtained from (A) WT mice injected intratracheally with vehicle, (B) WT mice injected intratracheally with lipopolysaccharide (LPS), (C) UTI (–/–) mice injected intratracheally with vehicle, (D) UTI (–/–) mice injected intratracheally with LPS. Twenty-four hours after injection, mice were sacrificed and assessed (n = 4 in each group). Original magnification ×200. (E) Quantitative analysis of neutrophil sequestration into the lung. Neutrophil infiltration was assessed by averaging the number of neutrophils enumerated in 30 randomly selected, high power fields (HPFs; ×400) in each slide. Open bars, WT mice; filled bars, UTI (–/–) mice. * $P < 0.01$ versus vehicle-treated mice, # $P < 0.01$ versus LPS-treated WT mice. Values are the mean plus or minus standard error of the mean in each group. HPF, high power field.

experimental groups 24 hrs after the intratracheal instillation. LPS treatment induced significant elevations of these cytokines and chemokines as compared with vehicle treatment in both genotypes of mice ($P < 0.01$; Table 2). In the presence of LPS treatment, the local expression of MCP-1 and KC was significantly higher in UTI (–/–) mice than in WT mice ($P < 0.05$ for KC, $P < 0.01$ for MCP-1). The expression of IL-1β and MIP-1α was not different between the two genotypes of mice in the presence of LPS (Table 2).

In the Absence of UTI, Gene Expression of Proinflammatory Cytokines, Chemokines, and ICAM-1 Are Able to Be Expressed at a Higher Level in Response to Endotoxin Injury. To investigate the role of UTI in the gene expression of proinflammatory cytokines, chemokines, and ICAM-1 related to bacterial endotoxin, we compared the gene levels for IL-1β, MIP-1α, MCP-1, KC, and ICAM-1 in the lung among the four experimental groups 4 hrs after the intratracheal instillation. Compared with vehicle treatment, LPS administration

Table 2. Protein Levels of Cytokines and Chemokines in the Lung Tissue Supernatants After Challenge with Lipopolysaccharide (LPS)[a]

Genotype	Challenge	IL-1β (pg/total lung supernatants)	MIP-1α (pg/total lung supernatants)	MCP-1 (pg/total lung supernatants)	KC (pg/total lung supernatants)
WT	Vehicle	85.9 ± 85.9	0	10.6 ± 4.9	4.67 ± 4.67
	LPS	18,287.6 ± 2236.0*	861.7 ± 140.6*	473.3 ± 41.9*	212.77 ± 18.75*
UTI (–/–)	Vehicle	190.9 ± 190.9	0	15.3 ± 6.3	49.76 ± 48.64
	LPS	21,416.0 ± 3661.9*	1035.0 ± 386.1*	752.9 ± 145.4*##	293.68 ± 51.93*#

[a] Lungs from mice were obtained 24 hrs after the administration of vehicle or LPS. Interleukin-1β (IL-1β), macrophage inflammatory protein-1α (MIP-1α), macrophage chemoattractant protein-1 (MCP-1), and keratinocyte chemoattractant (KC) levels in the lung tissue supernatants were measured by enzyme-linked immunosorbent assays. Results are mean plus or minus standard error of the mean (n = 10).
* $P < 0.01$ versus vehicle-treated mice.
# $P < 0.05$ versus LPS-treated WT mice.
$P < 0.01$ versus LPS-treated WT mice.

Table 3. mRNA Levels for Cytokines, Chemokines, and ICAM-1 in the Lung After Challenge with Lipopolysaccharide (LPS)[a]

Genotype	Challenge	IL-1β (relative intensity)	MIP-1 (relative intensity)	MCP-1 (relative intensity)	KC (relative intensity)	ICAM-1 (relative intensity)
WT	Vehicle	0.38 ± 0.72	0.62 ± 0.23	1.06 ± 0.25	0.69 ± 0.16	1.97 ± 0.31
	LPS	2.71 ± 0.63	5.00 ± 2.26	3.57 ± 1.33	9.71 ± 4.53	3.47 ± 0.63
UTI (–/–)	Vehicle	1.03 ± 0.44	0.33 ± 0.13	0.88 ± 0.27	1.16 ± 0.44	1.43 ± 0.32
	LPS	9.20 ± 2.44*#	14.32 ± 2.76*#	14.29 ± 3.19*#	30.13 ± 9.10*#	8.90 ± 1.20*##

[a] Lungs from mice were obtained 4 hrs after the administration of vehicle or LPS. IL-1β, MIP-1α, MCP-1, KC, and intercellular adhesion molecule-1 (ICAM-1) levels in the lung were measured by RT-PCR. The relative density of gene expression was quantitated by the standard method according to the manufacturer's protocol and normalized to 18S rRNA. Results are mean plus or minus standard error of the mean ($n = 5$).
* $P < 0.01$ versus vehicle-treated mice.
# $P < 0.05$ versus LPS-treated WT mice.
$P < 0.01$ versus LPS-treated WT mice.

resulted in elevated gene expression for these cytokines, chemokines, and ICAM-1 in both genotypes of mice, with statistical significance for UTI (–/–) mice ($P < 0.05$) and without statistical significance for WT mice except for KC ($P < 0.01$; Table 3). In the presence of LPS treatment, the local expression of IL-1β, MIP-1α, MCP-1, and ICAM-1 was significantly higher in UTI (–/–) mice than in WT mice ($P < 0.05$ for IL-1β, MIP-1α, MCP-1, and KC, $P < 0.01$ for ICAM-1).

Discussion

The present study has demonstrated that UTI (–/–) mice are more sensitive to acute lung injury induced by LPS than WT mice. The acute lung injury comprises neutrophilic inflammation, interstitial edema, and alveolar hemorrhage. This enhancement in acute lung injury is concomitant with the increased lung expression of MCP-1, KC, and ICAM-1 at levels of mRNA and/or proteins.

A number of mediators, including lipid mediators, cytokines, free radicals, complement fragments, coagulatory factors, and proteases contribute to the pathogenesis of ARDS and acute lung injury (4, 20). Among them, the products from neutrophils are recognized to play important roles. Activated neutrophils release various kinds of mediators, including proteases and oxygen radicals (21). Protease-antiprotease imbalance has been involved in inflammatory lung disease such as ARDS (22, 23), as well as inflammatory conditions at the other sites (24, 25). Because neutrophil elastase exerts the most injurious effects on many kinds of substrates (elastin, types I through IV collagen, fibronectin, laminin, and proteoglycans) among the proteases produced by neutrophils, it can be a key mediator of tissue injury (26). Furthermore, neutrophil elastase inhibitors inhibit lung injury in several animal models (27–29).

UTI is a multivalent Kunitz-type serine protease inhibitor that inhibits trypsin, α-chymotrypsin, plasmin, cathepsin G, leukocyte elastase, and matrix metalloproteases, as well as proteases in the coagulation cascade. Considering the possible roles of proteases in inflammatory diseases, UTI would be expected to have a beneficial effect on inflammatory disorders. In fact, intravenous administration of UTI reduces ischemia-reperfusion injury in the rat brain (14), improves impaired cardiac function during hemorrhagic shock in rats (15), and ameliorates clinical parameters including survival rate, cardiac index, and blood pressure during septic shock in canines (16). Another study has demonstrated that intraperitoneal administration of UTI has preventive effects on rat experimental glomerulonephritis with the inhibition of intraglomerular infiltration of inflammatory cells (17). These studies, however, have some limitations since the animals were treated with human UTI as a foreign protein. In the present study, we used a UTI-null mouse model to show a protective role of UTI in acute inflammation.

In our study, UTI (–/–) mice, as compared with WT mice, showed a significant increase in the lung water content and in the neutrophil numbers in BAL fluid in the presence of LPS. The results indicate that recruitment of water and inflammatory cells into the lung are more enhanced in LPS-challenged UTI (–/–) mice than in LPS-challenged WT mice. These results may suggest that UTI inhibits proteases that actually cause the lung injury and subsequent cascade of inflammatory events.

As well as the other serine type protease inhibitors, UTI reportedly has other anti-inflammatory properties in addition to protease inhibition. UTI inhibits LPS-induced production of TXB2 (10) and TNF-α in monocytes (12) and PHS-2 in polymorphonuclear leukocytes (13). Also, UTI inhibits LPS-induced IL-8 gene expression *in vitro* (11). IL-8 is thought to have a significant role in acute lung injury induced by LPS (30). In addition, it is likely that IL-1 and MIP participate in the development of acute lung injury (31, 32). Also, MCP-1 is a potential mediator in acute lung injury because anti-MCP-1 antibodies have reduced the injury (33). In our study, the gene expression for IL-1β, MIP-1α, and MCP-1 and the protein expression of MCP-1 and KC were significantly higher in UTI (–/–) mice than in WT mice in the presence of LPS. These results indicate that UTI can have protective effects on acute lung injury induced by LPS, at least partly, through inhibition of the enhanced expression of these cytokines and chemokines. On the other hand, it also can be speculated that in the absence of UTI,

the lung damage can be more severe, can be longer lasting, or perhaps can result in even more elevated levels of these cytokines. Future studies are needed to address these points.

ICAM-1 is an important ligand for the β2 integrin family of leukocyte membrane glycoproteins. Interactions between these complementary surface molecules mediate firm adhesion of neutrophils to the endothelial cell monolayers *in vitro* and into the interstitium *in vivo* (34). Indeed, anti–ICAM-1 antibodies inhibit both neutrophil sequestration and lung injury induced by complement activation, suggesting the protective role of ICAM-1 in the epithelial-endothelial integrity (35). UTI reportedly inhibits the expression of ICAM-1 during surgery or that induced by TNF-α (36) *in vitro*. In the present study, mRNA expression for ICAM-1 in the lung was concomitant with the increase in inflammation and neutrophil sequestration *in vivo*. Thus, our study could be the *in vivo* demonstration of a direct protective role of UTI in the local ICAM-1 expression during inflammation.

In conclusion, the present study has shown that UTI participates in the protection against acute lung injury induced by bacterial endotoxin. The effect is concomitant with the inhibition of local expression of proinflammatory cytokines, chemokines, and ICAM-1. These findings identify a new therapeutic application of UTI for acute lung injury in addition to shock, DIC, and pancreatitis, all of which are involved in systemic inflammatory response syndrome.

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