Inhibition of lipopolysaccharide-induced tissue factor expression in monocytes by urinary trypsint inhibitor in vitro and in vivo

Perenlei Molor-Erdene, Kenji Okajima, Hirotaka Isobe, Mitsuhiro Uchiba, Naoaki Harada, Nobuhiko Shimozawa, Hiroaki Okabe

Department of Diagnostic Medicine, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan

Summary

Tissue factor (TF) plays a critical role in the pathogenesis of disseminated intravascular coagulation (DIC) observed in patients with septic shock. Urinary trypsin inhibitor (UTI), a multivalent protease inhibitor, is currently used for treatment of patients with septic shock. This study was undertaken to determine whether UTI reduces LPS-induced coagulation abnormalities by inhibiting lipopolysaccharide (LPS)-induced expression of TF by monocytes. UTI inhibited LPS-induced increases in both TF activities and TF mRNA expression in monocytes without affecting the viability. Although activation of nuclear factor-κB (NF-κB), activator protein-1 (AP-1) and extracellular signal-regulated kinase (ERK)1/2 were shown to be critically involved in LPS-induced increases in TF activities in isolated monocytes, UTI inhibited phosphorylation of ERK1/2 and decreased expression of early growth response factor-1 (Egr-1) induced by LPS without affecting the activation of NF-κB and AP-1. UTI inhibited both the expression of TF mRNA in whole blood, increases in TF activities in mononuclear cells, and increases in serum levels of fibrin and fibrinogen degradation products (E) in rats given LPS without affecting the number of monocytes in the peripheral blood. Taken together these results strongly suggested that UTI might reduce LPS-induced coagulation abnormalities in rats by inhibiting TF expression in monocytes through inhibition of Egr-1 expression.

Keywords

Tissue factor, monocytes, Egr-1, disseminated intravascular coagulation

Introduction

Disseminated intravascular coagulation (DIC) is a serious complication of severe sepsis and septic shock (1). Since the expression of tissue factor (TF) on lipopolysaccharide (LPS)-stimulated monocytes plays a critical role in the pathogenesis of DIC observed in patients with septic shock (2–4), inhibition of TF expression on monocytes is potentially important for treatment of DIC associated with septic shock.

TF expression is regulated by various transcriptional factors including nuclear factor-κB (NF-κB), activator protein-1 (AP-1) and early growth response factor-1 (Egr-1) (5–8). NF-κB is a homo- or heterodimeric complex of proteins and the c-Rel/p65 heterodimer is critically involved in regulation of TF gene expression (5–7). In unstimulated monocytes, NF-κB is localized in the cytoplasm as an inactive form bound to IκB. Stimulation with LPS induces NF-κB activity causing dissociation and subsequent degradation of IκB proteins, allowing NF-κB dimers to enter the nucleus and induce gene expression (9).

Two AP-1 sites are located in the TF promoter region and these sites are required for maximal TF induction. The AP-1 family of transcription factors is divided into two groups: the Fos-related proteins such as c-Fos, and the Jun proteins such as c-Jun. AP-1 proteins are phosphorylated and activated by c-Jun N-terminal kinase (JNK) and p38, members of the mitogen-activated protein kinase (MAPK) family, in LPS-stimulated monocytes (10). JNK and p38 are activated by phosphorylation by the upstream activator MAPK kinases in response to LPS (11, 12).

The transcriptional factor Egr-1 is an 80 kDa nuclear phosphoprotein containing 3 zinc-finger DNA binding domains. The Egr-1 gene can be induced by diverse signals that initiate growth and differentiation without de novo protein synthesis as a requirement (13). Putative nucleotide recognition elements for Egr-1, which usually overlap with Sp1 binding sites, appear in the promoter of TF gene (5). Stimulation of THP-1 cells with LPS has been shown to induce Egr-1 expression via activation of extracellular signal-regulated kinase (ERK)1/2 (14).
Urinary trypsin inhibitor (UTI), also known as ulinastatin, is a Kunitz-type protease inhibitor found in human urine (15). UTI is synthesized from inter-α-trypsin inhibitor through proteolytic cleavage by neutrophil elastase at the site of inflammation (16). UTI inhibits various serine proteases such as trypsin, chymotrypsin, neutral elastase, and plasmin (17). According to the multivalent nature of protease inhibition, UTI is considered to prevent organ injury by inhibiting the activity of trypsin, chymotrypsin and neutrophil elastase (18, 19).

In addition to its inhibitory effects against proteases, UTI suppressed urokinase-type plasminogen activator production in tumor cells, thereby inhibiting the invasion of tumor cells. Although the precise mechanism(s) underlying this inhibition by UTI is not fully elucidated, inhibition of ERK1/2 activation by UTI might be critically involved in the inhibitory mechanism(s) (20). Since the activation of ERK1/2 has been demonstrated to activate serum response factor leading to subsequent Egr-1 expression, it is possible that UTI inhibits LPS-induced TF expression in monocytes by inhibiting Egr-1 expression through inhibition of ERK1/2 activation.

In the present study, we determined whether UTI reduces coagulation abnormalities by inhibiting TF production in monocytes stimulated with LPS and in rats administered LPS.

**Methods**

**Materials**
UTI is a generous gift from Mochida Pharmaceutical Co. Ltd. (Tokyo, Japan). UTI concentrates used in the present study revealed a single band and a single peak on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Sephadex gel chromatography, respectively (21). LPS (Escherichia coli, serotype 055:B5) was purchased from Sigma (St. Louis, MO). Polyclonal rabbit antibodies against human IkBα (#9242), JNK (#9252), phosphorylated JNK (Thr183/Tyr185) (#9251), p38 (#9212), phosphorylated p38 (Thr180/Tyr182) (#9211), ERK1/2 antibody (#9102), phosphorylated ERK1/2 antibody (Thr202/Tyr204) (#9101) and PD98059 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against Egr-1 and PU.1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-β-actin antibody was from Sigma. Factor VII deficient plasma was purchased from Kokusai-shiyaku (Kobe, Japan). MG-132 and SB203580 were purchased from Alexis (Basel, Switzerland). All reagents used were of analytical grade.

**Monocyte preparation and incubation**
Peripheral blood mononuclear cells were isolated fromuffy coats obtained from healthy volunteer blood donors as described previously (22). The cell preparations were >90% monocytes, as determined by May-Giemsa staining. Cell viability was >95%, as determined by trypan blue dye exclusion test. Mononuclear cells were adjusted to an appropriate volume and cultured in RPMI 1640 (Invitrogen, Grand Island, NY) plus 1% supplemented calf serum (Hyclone, Logan, UT) at 37°C in a humidified 5% CO₂ incubator with or without UTI for 30 min and then stimulated with LPS (100 ng/ml).

**Measurement of TF activity**
Human monocytes were scraped and analyzed for TF activity as described previously (23). Briefly, indicated times after LPS stimulation, human monocytes (1x10⁶ cells) were washed twice by phosphate buffered saline, and stored at ~80°C until TF activity measurement. After thawing, mononuclear cell pellets were sonicated for 30 sec to scrap the monocyte pellets, and then dissolved in 200 µl clotting buffer (12 mM sodium acetate, 7 mM diethylbarbiturate, and 130 mM sodium chloride). Fifty µl of suspended cells were mixed with 50 µl of citrated plasma, and clotting times were measured after recalcification with 50 µl of 20 mM CaCl₂ solution at 37°C. TF equivalents were determined by using a standard curve obtained from rabbit brain thromboplastin (Neoplastin Plus, Boehringer Mannheim, Mannheim, Germany). Preincubation of the cell suspension with a monoclonal antibody against TF blocked procoagulant activity by >90%. In addition, an assay performed with factor VII deficient plasma showed prolonged clotting times. These data indicate that the cellular procoagulant activity measured in this experiment results from the triggering of the extrinsic coagulation pathway by TF expressed in monocytes.

**Western blot analysis**
Various times after LPS stimulation, whole cell lysates of monocytes (2x10⁶ cells/assay) were collected as described previously (24). Samples containing equal amounts of protein were separated using SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Membranes were incubated with appropriate antibodies at 4°C overnight and subsequently with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Specific proteins were visualized using enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

**Detection of specific binding of c-Rel, p65 and c-Fos to DNA by enzyme-linked immunosorbent assay (ELISA)**
Human monocytes (1x10⁷ cells/assay) were stimulated with LPS for 1 h in the presence or absence of UTI. Nuclear extracts were prepared as described previously (24). Analysis of the specific binding of c-Rel, p65 and c-Fos to their consensus oligonucleotides was performed in nuclear extracts using the ELISA-based assay kits (TransAM, Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. This method is based on nonisotopic quantitative ELISA-based analysis and was reported to be more sensitive than electrophoretic mobility shift assay (25).

**Animal model of LPS-induced coagulation abnormalities**
The study protocol was approved by the Kumamoto University School of Medicine Animal Care and Use Committee, and the care and handling of the animals were in accordance with the guidelines of the National Institutes of Health. Specific pathogen-free male Wistar rats (220 g to 280 g, Kyudo, Kumamoto, Japan) were given water but not food for 16 hours before the experiments. LPS (5 mg/kg) were administered intravenously via the tail vein. Thirty nM/kg of UTI is clinically used for treatment of patients with shock, and the plasma level of UTI reached 900
nM in humans after administration of 30 nM/kg of UTI (26). In rats, the plasma level of UTI reached about 1,000 nM after administration of 300 nM/kg of UTI (27). To attain plasma levels of UTI in rats similar to those in humans, we administered UTI at a dose of 300 nM/kg in the present study. UTI (300 nM/kg) was injected 30 min before the administration of LPS. Control animals were treated similarly except that they received saline instead of LPS. Indicated times after LPS administration, rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) and were exsanguinated via the abdominal aorta. Blood samples were collected in tubes containing 1:10 volume of 3.8% (wt/vol) sodium citrate. Serum levels of fibrin and fibrinogen degradation products E [FDP(E)] were measured by latex agglutination assay as previously described (28).

**Mononuclear cell preparation from LPS-treated rats**
Six hours after LPS administration, mononuclear cells were isolated using Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) from citrated blood samples as described previously (22). TF activities of mononuclear cells were determined as described above.

**Quantitative mRNA analysis**
RNA was extracted from cultured human monocytes or rat whole blood samples using TRIzol (Life Technologies, Gaithersburg, MD) reagent according to manufacturer’s instruction. This yielded 10 to 12 μg of total RNA from 5x10^6 cells of cultured human monocytes and 5 to 30 μg of total RNA from 1 ml of rat blood. RNA samples were diluted to a final concentration of 0.2 μg/μl in RNase-free water and stored at −80°C until use. Synthesis of cDNA was performed with 1 μg of total RNA by using TaqMan reverse transcription reagents (Applied Biosystems, Branchburgh, NJ). The 20 μl reverse transcription (RT) reaction consisted of 1 x TaqMan RT buffer, 5.5 mM magnesium chloride, 500 μM deoxyNTPs Mixture, 2.5 μM randomhexamers, 0.4 U/μl RNase inhibitor, and 1.25 U/μl MultiScribe reverse transcriptase. Thermal cycling parameters for reverse transcription were 10 min hold at 25°C, 30 min hold at 48°C and 5 min hold at 95°C. Real-time PCR amplification and determination were done using the ABI PRISM 7000 Sequence Detection System, TaqMan Universal PCR Master Mix and commercially available pre-designed, gene-specific primers and FAM-labeled probe sets for quantitative gene expression (TaqMan Gene Expression Assays; human TF, code Hs00175225_m1 and rat TF, code Rn00564925_m1; and TaqMan Endogenous Controls; human GAPDH, code 4310884E; and rat GAPDH, code 4352338E) (Applied Biosystems). All the probes used in this experiments span an exon-intron boundary. TF and GAPDH mRNA were

![Figure 1. Effects of urinary trypsin inhibitor (UTI), MG-132, SB203580 and PD98059 on LPS-induced increases in TF activities in monocytes. (A) TF activities were determined in isolated human monocytes stimulated with LPS (100 ng/ml) at the indicated time points after LPS stimulation. TF activities in human monocytes were measured using the clotting assay method as described in Methods. Data are expressed as mean ± SD of three samples in one experiment representative of three performed with similar results. *, p<0.01 versus time 0. (B) Human monocytes preincubated with various concentrations of UTI for 30 min were stimulated with LPS (100 ng/ml). TF activities were determined at 6 hours after LPS stimulation. Data are expressed as mean ± SD of three samples in one experiment representative of three performed with similar results. *, p<0.01 versus control; †, p<0.05 versus LPS without UTI. (C) Human monocytes preincubated with UTI (6 μM), MG-132 (MG, 1 μM), SB203580 (SB, 1 μM) and PD98059 (PD, 10 μM) for 30 min were stimulated with LPS (100 ng/ml). TF activities were determined at 6 hours after LPS stimulation. Data are expressed as mean ± SD of three samples in one experiment representative of three performed with similar results. *, p<0.01 versus control; †, p<0.05 versus LPS without UTI or various inhibitors.](https://www.thrombosis-online.com/.../1001066444.../54.70.40.11)
Figure 2. Effect of UTI on increases in TF mRNA levels in monocytes stimulated with LPS. (A) TF mRNA levels in human monocytes were determined at indicated time points after stimulation with LPS (100 ng/ml). TF mRNA levels in human monocytes were detected by real time RT-PCR as described in Methods. Data are expressed as mean ± SD of three samples in one experiment representative of three performed with similar results. *, p < 0.01 versus time 0. (B) TF mRNA levels in human monocytes preincubated with UTI (6 µM) for 30 min were determined at 1 hour after stimulation with LPS (100 ng/ml). Data are expressed as mean ± SD of three samples in one experiment representative of three performed with similar results. *, p < 0.01 versus control; †, p < 0.05 versus LPS.

Figure 3. Effect of UTI on degradation of IκBα and increases in the DNA-binding activities of c-Rel and p65. (A) Intracellular levels of IκBα were determined in human monocytes in the absence or presence of UTI (6 µM) using Western blot analysis at indicated time points after LPS (100 ng/ml) stimulation. UTI was added 30 min before LPS stimulation. Intracellular levels of β-actin in monocytes were measured as the loading control. Data are expressed as mean ± SD of three samples in one experiment representative of three performed with similar results. Open circles: LPS without UTI; closed circles: LPS with UTI. *, p < 0.01 versus time 0. (B) Human monocytes preincubated with UTI (6 µM) for 30 min were stimulated with LPS (100 ng/ml) for 60 min. DNA-binding activities of c-Rel and p65 were determined using the ELISA-based method as described in Methods. Data are expressed as mean ± SD of three samples in one experiment representative of three performed with similar results. *, p < 0.01 versus control; n.s., not significant versus LPS.
quantified by parallel estimation. The thermal cycler conditions were 2 min hold at 50°C, 10 min hold at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

Data analysis

Data are presented as means ± SD. The results were compared using analysis of variance and Scheffe’s post hoc test for multiple comparison or unpaired t-test for single comparison. A level of p < 0.05 was accepted as statistically significant.

Results

Effect of UTI on LPS-induced increases in TF activities in isolated monocytes in vitro

To determine whether UTI inhibits LPS-induced TF expression on monocytes, we examined the effect of UTI on LPS-induced increases in TF activities in isolated human monocytes in vitro. TF activities in monocytes began to increase 2 hours after LPS stimulation, reaching a maximum at 6 hours, and thereafter gradually decreased (Fig. 1A). Preincubation with UTI for 30 min inhibited the LPS-induced increases in TF activities in monocytes observed at 6 hours after stimulation in a concentration-dependent manner (Fig. 1B). LPS-induced increases in TF activities were inhibited by MG-132, an inhibitor of the NF-κB pathway, by SB203580, an inhibitor of the p38 pathway, and by PD98059, an inhibitor of the ERK1/2 pathway (Fig. 1C). To determine whether UTI has cytotoxic effects on monocytes, we measured the total cell number and cell viability using the trypan blue dye exclusion test at 6 hours after incubation with 0 to 6 μM of UTI and/or LPS (100 ng/ml). Neither the total cell number nor cell viability were affected by incubation with UTI in the presence of LPS (data not shown), suggesting that UTI might not be cytotoxic to monocytes in the present experimental conditions.

Inhibition of LPS-induced increases in TF mRNA level in monocytes by UTI in vitro

Intracellular levels of TF mRNA increased after LPS stimulation, reaching the maximum level at 1 hour after the stimulation, and gradually decreased thereafter (Fig. 2A). The amount of GAPDH mRNA did not change with LPS treatment (data not shown). Preincubation with UTI at a concentration of 6 μM for 30 min significantly inhibited the LPS-induced increases in TF mRNA levels observed at 1 hour after LPS stimulation (Fig. 2B).

Effect of UTI on LPS-induced activation of NF-κB in human monocytes in vitro

To determine whether UTI inhibits LPS-induced activation of NF-κB, we examined effect of UTI on LPS-induced degradation of IkBα in isolated monocytes in vitro. As shown in Figure 3A, the intracellular levels of IkBα began to decrease at 5 min after LPS stimulation, reaching a minimum level at 30 min, and then increased gradually. Preincubation with UTI for 30 min did not inhibit LPS-induced degradation of IkBα (Fig. 3A). In addition, preincubation with UTI for 30 min did not inhibit LPS-induced increases in DNA-binding activities of c-Rel and p65 to their consensus oligonucleotides (Fig. 3, B and C).

Effect of UTI on LPS-induced activation of AP-1 in human monocytes in vitro

To determine whether UTI inhibits LPS-induced activation of AP-1, we examined the effect of UTI on LPS-induced phosphorylation of JNK and p38 in isolated monocytes in vitro. Intracellular levels of phosphorylated JNK increased after LPS stimulation, reaching the maximum level at 15 min after stimulation, and gradually decreased thereafter (Fig. 4A). Preincubation with UTI for 30 min did not inhibit LPS-induced phosphorylation of JNK observed at 15 min after LPS stimulation (Fig. 4A). The cellular levels of phosphorylated p38 were increased by LPS stimulation, reaching a maximum value at 15 min, and decreased thereafter (Fig 4B). Preincubation with UTI for 30 min did not inhibit LPS-induced phosphorylation of p38 in monocytes at 15 min after LPS stimulation (Fig 4B). In addition, preincubation with UTI for 30 min did not inhibit LPS-induced increases in the DNA-binding activities of c-Fos (Fig. 4C).

Effect of UTI on expression of Egr-1 in monocytes stimulated with LPS

To determine whether UTI inhibits LPS-induced activation of Egr-1, we examined the effect of UTI on LPS-induced increases in Egr-1 levels in isolated monocytes in vitro. Intracellular levels of Egr-1 increased after LPS stimulation, peaking at 30 min after stimulation, and decreased thereafter (Fig. 5). Preincubation with UTI for 30 min inhibited the increase in Egr-1 expression in monocytes stimulated with LPS (Fig. 5).

Effect of UTI on LPS-induced phosphorylation of ERK1/2 in monocytes in vitro

To determine whether UTI inhibits LPS-induced activation of ERK1/2, thereby suppressing Egr-1 expression, we examined the effect of UTI on LPS-induced phosphorylation of ERK1/2 in isolated monocytes in vitro. Intracellular levels of phosphorylated ERK1/2 began to increase at 5 min after LPS stimulation, peaking at 15 min after stimulation, and decreased thereafter (Fig. 6). Preincubation with UTI for 30 min inhibited the LPS-induced phosphorylation of ERK1/2 in monocytes at 15 min after stimulation (Fig. 6).

Effect of UTI on the expression of TF mRNA in the whole blood and serum levels of FDP(E) in rats given LPS

To determine whether UTI inhibits TF expression in rats given LPS, we investigated the effect of UTI on LPS-induced increases in TF mRNA levels in whole blood of rats given LPS. TF mRNA levels in the whole blood increased after LPS administration, reaching a maximum level at 90 min after LPS administration, and gradually decreased thereafter (Fig. 7A). UTI inhibited increases in levels of whole blood TF mRNA observed at 90 min after LPS administration (Fig. 7B). UTI inhibited increases in TF activities of mononuclear cells observed at 6 hours after LPS administration (Fig. 7C). When rats were intravenously administered LPS, serum levels of FDP(E) began to increase at 3 hours after the administration and continued to increase until 6 hours after administration (29). UTI significantly inhibited increases in serum levels of FDP(E) observed at 6 hr after LPS administration (Fig. 8).
Figure 4. Effect of UTI on increases in intracellular levels of phosphorylated JNK, phosphorylated p38 and the DNA-binding activities of c-Fos in monocytes stimulated with LPS. (A, B) Human monocytes were stimulated with LPS (100 ng/ml). Intracellular levels of phosphorylated JNK (p-p54 and p-p46) and phosphorylated p-38 (p-p38) were determined using Western blot analysis in monocytes in the absence or presence of UTI (6 µM) at indicated time points after LPS stimulation. UTI was added 30 min before LPS stimulation. Intracellular levels of non-phosphorylated JNK (p54 and p46) and non-phosphorylated p-38 (p38) were determined as the loading control. Data are expressed as mean ± SD of three samples in one experiment representative of three performed with similar results. Open circles: LPS without UTI; closed circles: LPS with UTI. *, p < 0.01 versus time 0. (C) DNA-binding activities of c-Fos were measured using the ELISA-based method in human monocytes preincubated with UTI (6 µM) at 60 min after incubation with LPS (100 ng/ml). UTI was added 30 min before LPS stimulation. Data are expressed as mean ± SD of three samples in one experiment representative of three performed with similar results. *, p < 0.01 versus control; n.s., not significant versus LPS.
Effect of UTI on the numbers of total leukocytes, lymphocytes and monocytes in rats given LPS
Although the numbers of total leukocytes, lymphocytes and monocytes were decreased by LPS administration at 90 min and 6 hours after LPS administration, UTI did not affect LPS-induced changes in the numbers of these cells (Table 1).

Discussion
In the present study, we demonstrated that UTI inhibited both LPS-induced increases in TF activities and TF mRNA levels in monocytes stimulated with LPS in vitro and the increases in TF mRNA levels in the whole blood of rats given LPS in vivo. In addition, UTI inhibited LPS-induced increases in serum levels of FDP(E) in rats. These results strongly suggested that UTI might inhibit LPS-induced coagulation abnormalities in rats by suppressing LPS-induced TF expression in monocytes. UTI has been shown to increase expression of TF in neutrophils and endothelial cells as well as monocytes (30–32). Thus, it is possible that UTI also inhibits TF expression in these cells other than monocytes in rats given LPS, contributing to reduction of LPS-induced coagulation abnormalities in the present study.

In the present study, UTI inhibited LPS-induced TF expression in a dose dependent fashion in monocytes in vitro and, thus, UTI at a concentration of 6 µM, the concentration that showed maximum inhibition of TF expression, was employed to analyze the molecular mechanism(s) underlying this inhibitory effect in vitro. Since 6 µM of UTI did not affect cell viability of monocytes at 6 hours after incubation, it is less likely that the inhibitory effect of UTI on LPS-induced TF expression in monocytes is due to its cytotoxic effect.

The concentration of UTI in rat plasma is reported to be 2.5 µM and only 45 nM is the free form capable of exerting biological activity (33). Although we did not measure plasma concentration of UTI in rat plasma in the present study, Ohzawa et al. (27) reported that the concentration of UTI was increased to...
Figure 7. Effect of UTI administration on increases in TF mRNA levels in the whole blood and TF activities of mononuclear cells in rats given LPS. (A) LPS (5 mg/kg) was administered intravenously. TF mRNA expression in whole blood was measured at indicated time points after LPS administration. Data are expressed as mean ± SD of three samples in one experiment representative of three performed with similar results. *, p < 0.01 versus time 0. (B) UTI (300 nM/kg, iv) was administered 30 min before intravenous administration of LPS (5 mg/kg). TF mRNA levels in whole blood and TF activities of mononuclear cells were determined at 90 min and 6 hours after LPS administration, respectively. Data are expressed as mean ± SD of three animals. p < 0.01 versus control; †, p < 0.05 versus LPS plus saline.

Figure 8. Effect of UTI administration on LPS-induced increases in serum levels of FDP(E) in rats. UTI (300 nM/kg, iv) was administered 30 min before intravenous administration of LPS (5 mg/kg). Serum levels of FDP(E) were determined at 6 hours after LPS administration by the latex agglutination method. Data are expressed as mean ± SD of three animals. *, p < 0.01 versus control; †, p < 0.05 versus LPS plus saline.

TF expression is regulated by various transcriptional factors including NF-κB, AP-1 and Egr-1 (5–8). In the present study, we demonstrated that LPS-induced TF expression was inhibited by MG-132, an inhibitor of the NF-κB pathway, or by SB 203580, an inhibitor of p38, suggesting that both the NF-κB and AP-1 pathways might be critically involved in LPS-induced expression of TF in monocytes in the present study. The activity of NF-κB is primarily regulated by its sequestration in the cytosol through anchoring to the inhibitory IκB proteins (9). As shown in the present study, UTI did not inhibit the degradation of IκBα and the binding of either c-Rel or p65 to target sites. These results suggested that the inhibitory effect of UTI on TF production in LPS-stimulated monocytes is not mediated by inhibition of the NF-κB pathway. These results are consistent with observations by Aosasa et al. (34) demonstrating that UTI did not inhibit LPS-induced activation of NF-κB in monocytes.

AP-1 is another important factor for regulating TF expression (5–8). Activation of JNK and p38 by phosphorylation was shown to enhance the transcriptional activity of AP-1 (10). In the present study, UTI did not inhibit LPS-induced phos-
phorylation of JNK and p38, and the increase in DNA-binding activity of c-Fos to its consensus sequence, suggesting that the inhibitory effect of UTI on TF expression might not be due to inhibition of AP-1 activation.

UTI inhibited the LPS-induced increase in intracellular levels of Egr-1 in the present study. Since Egr-1 is critically involved in TF expression of monocytes stimulated with LPS (14), UTI might inhibit TF expression by inhibiting activation of Egr-1 in monocytes stimulated with LPS.

Egr-1 gene expression is regulated by various transcriptional factors, including Elk-1 and Sap1a. Guha et al. (14) reported that Elk-1, activated by phosphorylated-ERK1/2, plays an important role in Egr-1 expression induced by LPS. They also reported that an inhibitor of the ERK1/2 pathway reduced LPS-induced TF expression via inhibition of Egr-1 expression in monocytes. LPS-induced TF expression in monocytes was inhibited by PD98059, an inhibitor of the ERK1/2 pathway, as shown in the present study, suggesting that activation of the ERK1/2 pathway might be critically involved in the LPS-induced TF expression in monocytes in the present study. Since UTI inhibited the LPS-induced phosphorylation of ERK1/2 in the present study, it is possible that UTI inhibits expression of Egr-1 by inhibiting ERK1/2 activation, thereby suppressing the expression of TF in monocytes stimulated with LPS. However, since other signaling pathways such as that involving CBP/P300, a transcriptional coactivator which enhances p65 activity (35), and Akt/P3K (36) play critical roles in TF expression, it is possible that UTI modulates LPS-induced TF expression by affecting pathways other than the ERK1/2-Egr-1 pathway. These possibilities should be examined by further studies in the near future.

Egr-1 plays a critical role in tumor necrosis factor-α (TNF-α) production in monocytes stimulated by LPS (14, 37). TNF-α contributes to the pathogenesis of coagulation abnormalities in sepsis by increasing TF expression on monocytes (38) and reducing the endothelial anticoagulant potential by decreasing thrombomodulin expression (39) and heparin-like glycosaminoglycans. (40). Our preliminary experiments showed that UTI inhibited TNF-α production in isolated human monocytes stimulated with LPS by inhibiting Egr-1 expression. Therefore, UTI might inhibit coagulation abnormalities in rats given LPS by inhibiting TNF-α expression as well as TF production in monocytes.

UTI was shown to be capable of inhibiting thrombin and factor Xa directly (21, 41). Thus, although inhibition of monocyte TF expression by UTI might at least partly explain its anti-coagulatory effect seen in rats given LPS in the present study, it is possible that UTI reduces LPS-induced coagulation abnormalities via direct inhibition of thrombin and factor Xa in rats. However, this possibility seems less likely since 1 μM of UTI, the expected plasma concentration in rats administered 300 nM/kg of UTI (27), did not inhibit these coagulation factors in vitro (21, 41).

In physiological conditions, the majority of UTI in plasma is present as a component of an inter-α-trypsin inhibitor and pre-α-trypsin inhibitor and UTI lacks some of its known activities in such precursor forms (42). UTI is released by neutrophil elastase in pathological conditions such as inflammation and thrombosis (43). It is likely that UTI released at the site of thrombus formation inhibits the expression of TF, thereby regulating the coagulation system. Consistent with this hypothesis was the report by Jourdain et al. (44) demonstrating that administration of inter-α-trypsin inhibitor reduced LPS-induced coagulation abnormalities in pigs.

TF is considered to be pro-inflammatory since anti-TF antibody reduced the mortality of baboons challenged with Escherichia coli by preventing inflammatory responses including hypotension and coagulation abnormalities (45). TF might enhance inflammatory responses by eliciting a variety of intracellular signaling events that may be implicated in inflammatory responses through interaction with protease activated receptor-1 and –2 on monocytes and endothelial cells (46). Consistent with this hypothesis is a recent report by Pawlinski et al. (32) demonstrating that inhibition of monocyte TF expression in endotoxemic mice decreased both coagulation abnormalities and inflammatory responses, thereby reducing the mortality of mice challenged with LPS. These observations strongly suggested that inhibition of coagulation abnormalities as well as inflammatory responses might be critical in the treatment of patients with severe sepsis and, thus, UTI might be a potential therapeutic agent in severe sepsis.

### Table 1: The numbers of leukocytes, lymphocytes, and monocytes in peripheral blood at 90 min (A) and 6 hours (B) after LPS administration in rats.

<table>
<thead>
<tr>
<th></th>
<th>No. of Leukocytes (x10³)</th>
<th>Lymphocytes (x10³)</th>
<th>Monocytes (x10³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.156 ± 1.343</td>
<td>6.959 ± 0.117</td>
<td>421 ± 185</td>
</tr>
<tr>
<td>LPS</td>
<td>1.533 ± 0.320*</td>
<td>1.345 ± 0.252*</td>
<td>88 ± 14*</td>
</tr>
<tr>
<td>LPS+UTI</td>
<td>1.300 ± 0.162**</td>
<td>1.145 ± 0.79**</td>
<td>75 ± 17*</td>
</tr>
<tr>
<td>(B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.914 ± 1.673</td>
<td>6.167 ± 0.940</td>
<td>351 ± 165</td>
</tr>
<tr>
<td>LPS</td>
<td>5.366 ± 1.236*</td>
<td>1.207 ± 0.387*</td>
<td>130 ± 63*</td>
</tr>
<tr>
<td>LPS+UTI</td>
<td>5.264 ± 1.461**</td>
<td>1.161 ± 0.591**</td>
<td>141 ± 80*</td>
</tr>
</tbody>
</table>

* p < 0.01 versus Control; n.s., not significant versus LPS.
References

17. Nishiyama T, Aibiki M, Hanaoka K. The effects of ucinastatin, a human protease inhibitor, on the trans-