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KUNITZ-TYPE TRYPSIN INHIBITOR PREVENTS LPS-INDUCED INCREASE OF CYTOSOLIC FREE Ca^{2+} IN HUMAN NEUTROPHILS AND HUVEC CELLS

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The protease inhibitor part of inter-α trypsin inhibitor is identical to urinary trypsin inhibitor (UTI). Preincubation of neutrophils and HUVEC cells with UTI inhibited increase of cytosolic free Ca^{2+} induced by LPS. Increase of cytosolic free Ca^{2+} induced by LPS in the presence of EGTA was also inhibited by UTI. In contrast, UTI did not inhibit increase of cytosolic free Ca^{2+} in cells stimulated by Ca^{2+} ionophore with or without EGTA. The effects of nine synthetic peptides of UTI on the concentration of cytosolic free Ca^{2+} in the neutrophils induced by LPS were examined. Preincubation with a peptide of UTI domain 2, NLPIVRGPCRAFIQL (83-97), was completely inhibited by the increase of cytosolic free Ca^{2+} in neutrophils. This region is identical to the trypsin inhibitor site of UTI. We propose that a function of UTI other than as a protease inhibitor is in regulation of intracellular Ca^{2+} and that this is due to its trypsin inhibitor region.

A Kunitz-type trypsin inhibitor is present in human serum and urine. It is identical to HI-30 (1) or urinary trypsin inhibitor (UTI) and is a heat- and acid-stable glycoprotein (2-3). Its precursor is inter-α trypsin inhibitor (ITI). It is linked to the C-terminal of α_1- microglobulin via two basic amino acids within the same reading frame (4-5). UTI inhibits various proteases (6). Recently ITI (UTI) family proteins have been thought to have important physiological roles besides as protease inhibitors such as in regulation of cell growth and cell stability (7-8). Compared with the concentrations of many other trypsin inhibitors in the circulation such as α_1 antitrypsin and α_2 macroglobulin, those of UTI and ITI are relatively high. Therefore, it has been thought that UTI might have some other function besides as a protease inhibitor. Recently we discovered that UTI significantly decreased the fluorescence of fura-2 loaded amniotic membranes by effects on interleukin-1β, TNF-α and LPS (9). From these findings, we speculated that UTI inhibits Ca^{2+}

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The abbreviations used are: UTI, urinary trypsin inhibitor; ITI, inter-α trypsin inhibitor; LPS, lipopolysaccharide.

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influx or intracellular Ca^{2+} mobilization. Here we report that UTI suppresses Ca^{2+} influx into neutrophils and HUVEC cells stimulated by LPS.

MATERIALS AND METHODS

UTI, α1 antitrypsin and cells: UTI was purified from human urine by the method of Proksch (10). Purified UTI, which migrated as a single band (45Kd) on SDS-PAGE and on Sephadex gel chromatography, was kindly provided by Mochida Pharmaceutical Co. Japan. α_1 Antitrypsin was provided by Midori Juji Co. Japan. Neutrophils (11) and HUVEC (human umbilical cord vein endothelial cell) cells (12) were prepared as described previously. Briefly, 50 ml of fresh human blood obtained from four healthy donors was mixed with 5,000 units of heparin and promptly centrifuged through Ficoll-Hypaque. The cell pellet was suspended in 0.16 M NH_4Cl containing 12 mM $NaHCO_3$ and 0.1 mM EDTA, pH 7.3, to lyse residual erythrocytes. HUVEC cells were cultured on a thin cover glass in a CO_2 incubator. Serum-free RPMI 1640 medium (Nissui, Japan) was used for neutrophils and HUVEC cells. Neutrophils and HUVEC cells were washed with PBS, and incubated in 1 ml of serum-free medium for 30 min in a CO_2 incubator before experiments.

Peptides: Nine UTI peptides; EVTKKEDS-(1A), MGMTSRYFYNGTSMA-(1B), NLPIVRGPCRAFIQLWAFDAVKGK-(2B), AVKGKCVLFPYG-(2C), NGNKFYSEKECREY-(2D), RGPCRAFI-(2A-1), RGP-(2A-2) and RAF-(2A-3) and the variant form RGPCAAFI-(2A-1v) (Fig.1) were synthesized by standard solid phase methods and purified by C-4 HPLC using an acetonitrile gradient in 0.1% trifluoroacetic acid. Analytical C-4 HPLC monitored at 220 nm showed less than 2% impurity in all samples. The identity of the peptides was monitored at 400-MHz or 250-MHz. The lyophilized synthetic polypeptides were dissolved in distilled water.

Measurement of cytosolic free Ca^{2+}: Digital imaging microscopy was carried out as described previously with some modifications (13). Briefly, cells were first incubated with 4 μg/ml fura-2 AM (Kumamoto, Japan) for 1 h. Before the experiment, the cells were washed with PBS, pH 7.3, and serum-free RPMI 1640 medium was added for 1 h at 37 °C. Fura-2 loaded cells on a

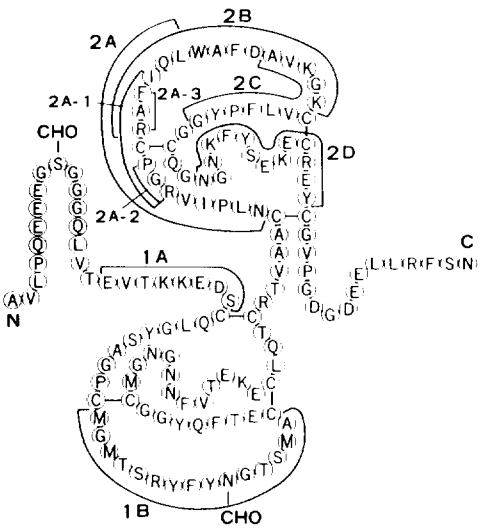

Fig. 1. Structure of UTI and its synthetic peptides. UTI has two Kunitz-type protease inhibitor domains. Synthetic peptides **1A** to **2D** are shown. **1A** and **1B** are parts of domain 1, and **2A**, **2A-1**, **2A-2**, **2A-3**, **2B**, **2C** and **2D** are parts of domain 2.

thin cover glass were placed on a microscope stage warmed to 37 °C. Cells in discs were preincubated with or without UTI (0.01-10 μM) for 10 min and then 0.1 mg/ml LPS or 0.01 mM A23187 was added. Cells in discs were also preincubated with or without 10 μM α_1 antitrypsin for 10 min and then 0.1 mg/ml LPS or 0.01 mM A23187 was added. Cytosolic free calcium ($[Ca^{2+}]_i$) was measured every 30 s for 390 s. The microscopic system consisted of an inverted microscope (Nikon TMD-EFQ, Japan) with a 100 x UV objective (Olympus, Japan). The cells were excited by ultraviolet light at 340 nm and 380 nm. Sequential images were collected through a single broad band pass filter (500 nm, band width 20 nm) at intervals of 30 s. The light source, a DC-stabilized Xenon lamp, was fitted with a computer-assisted device to alternate filters for the two wave lengths. Video images were acquired with a silicon-intensified target camera (2400-08, Hamamatsu Photonics K.K., Japan). The out-put was digitized with a color image analyzer ARGUS 100 (Hamamatsu Photonics K.K., Japan). The images were integrated to improve the S/N ratio and calculated as 340/380 nm ratio images. Finally, to detect the effect of UTI on intracellular Ca^{2+} mobilization, we added 2 nM EGTA to RPMI medium containing the cells. Then the cells were preincubated with UTI (10 μM) for 10 min at 37 °C and LPS or A23187 was applied. The change of $[Ca^{2+}]_i$ was measured similarly.

Effects of UTI peptides on $[Ca^{2+}]_i$ were also examined similarly. Neutrophils in each disc were preincubated with or without each peptide at 10 μM concentration for 10 min and then 0.1 mg/ml of LPS was added. $[Ca^{2+}]_i$ was measured every 30 s for 390 s.

To determine the $[Ca^{2+}]_i$, we made a calibration curve in our optical set up according to a previous report (14). $[Ca^{2+}]_i$ in all experiments was calculated from the fluorescence ratio values using the calibration curve. In all experiments, $[Ca^{2+}]_i$ was measured at the single cell level and means ± SD for 10 cells in repeated experiments (> 6 times) are presented in graphs. Statistical analysis were performed by Student's t test.

RESULTS

Effects of UTI on cytosolic Ca^{2+}: We measured the $[Ca^{2+}]_i$ of single cells. Unstimulated neutrophils and HUVEC cells with or without UTI showed $[Ca^{2+}]_i$ values of 88-110 nM and 78-94 nM, respectively. Both LPS and A23187 increased cytosolic Ca^{2+} time-dependently, the peak of concentrations was at about during 60-90 s (data not shown). Table 1 shows results on $[Ca^{2+}]_i$ at 60 s. Preincubation with 10 μM UTI inhibited the increase in cytosolic Ca^{2+} induced by LPS significantly, but did not inhibit the increase of cytosolic Ca^{2+} induced by A23187 (Table 1). In the presence of EGTA, LPS also increased cytosolic Ca^{2+} at 60 s, but A23187 did not. UTI inhibited the induction of cytosolic Ca^{2+} induced by LPS significantly in the presence of EGTA (Table 1). Thus intracellular Ca^{2+} mobilization was also inhibited by UTI. α_1 Antitrypsin did not suppress both LPS- or A23187-induced increase of $[Ca^{2+}]_i$. The increase of $[Ca^{2+}]_i$ induced by LPS was inhibited by UTI dose-dependently in neutrophils and HUVEC cells (Fig. 2 A, 2 B). Ca^{2+} influx was responsible for two-thirds of the total increase of $[Ca^{2+}]_i$ and intracellular Ca^{2+} mobilization for one-third.

Effects of peptides on cytosolic Ca^{2+}: We also measured the $[Ca^{2+}]_i$ of neutrophils stimulated by LPS in the presence of various UTI peptides. The $[Ca^{2+}]_i$ values of neutrophils at 60 s are shown in Fig. 3. 2A and 2A-1 inhibited the increase in $[Ca^{2+}]_i$ similarly to UTI, and 2B and 2A-3 inhibited it partially. However, the other peptides did not suppress the increase in $[Ca^{2+}]_i$. The variant form 2A-v (RGPCAAFI), in which 92-Arg was converted to Ala, did not inhibit the increase in $[Ca^{2+}]_i$ completely.

Table 1. Levels of $[Ca^{2+}]_i$ in neutrophils and HUVEC cells on various treatments

	Neutrophils nM (mean ± SD)	HUVEC cells nM (mean ± SD)
EGTA (-)		
LPS	450 ± 68	343 ± 70
UTI+LPS	105 ± 29*	92 ± 37*
α1 antitrypsin +LPS	483 ± 85	366 ± 50
EGTA (+)		
LPS	220 ± 35	160 ± 32
UTI+LPS	101 ± 46*	89 ± 22*
α1 antitrypsin +LPS	215 ± 69	163 ± 55
EGTA (-)		
A23187	532 ± 61	340 ± 58
UTI+A23187	540 ± 70	314 ± 49
α1 antitrypsin +A23187	547 ± 92	332 ± 67
EGTA (+)		
A23187	102 ± 29	90 ± 35
UTI+A23187	110 ± 23	96 ± 16
α1 antitrypsin +A23187	120 ± 20	86 ± 22

The cytosolic free Ca^{2+} concentration was measured 60 s after stimulation. Values are means ± SD for 5 replicate experiments. * $P<0.01$.

DISCUSSION

LPS, a membrane component of gram-negative bacteria, is a potent stimulator of many mammalian cells, and has multiple effects including the induction of secretion of inflammatory mediators such as IL-1, TNF-α and IL-8. LPS is reported to activate the platelet-activating factor (PAF) receptor directly and transduce Ca^{2+} signaling (15-16). LPS is known to increase intracellular calcium, a dynamic second messenger. On the other hand, the calcium ionophore A23187 is thought to form artificial membrane pores that could not be blocked by UTI. Preincubation with UTI inhibited the LPS-induced increase of $[Ca^{2+}]_i$ but did not inhibit the A23187-induced increase of $[Ca^{2+}]_i$. These results suggest that the cell membrane may be the site of action of UTI and that the effect of UTI may be due to inhibition of Ca^{2+} influx or mobilization.

Tobias and his collaborators (17,19-21) discovered that the activation of human endothelial and epithelial cells by LPS is mediated by LPS binding protein (LBP) and soluble CD14. Another form of CD14, a glycosyl-phosphatidyl-inositol-anchored membrane glycoprotein, is an endotoxin receptor on leukocytes (19-21). The mechanism of Ca^{2+} influx into neutrophils and endothelial

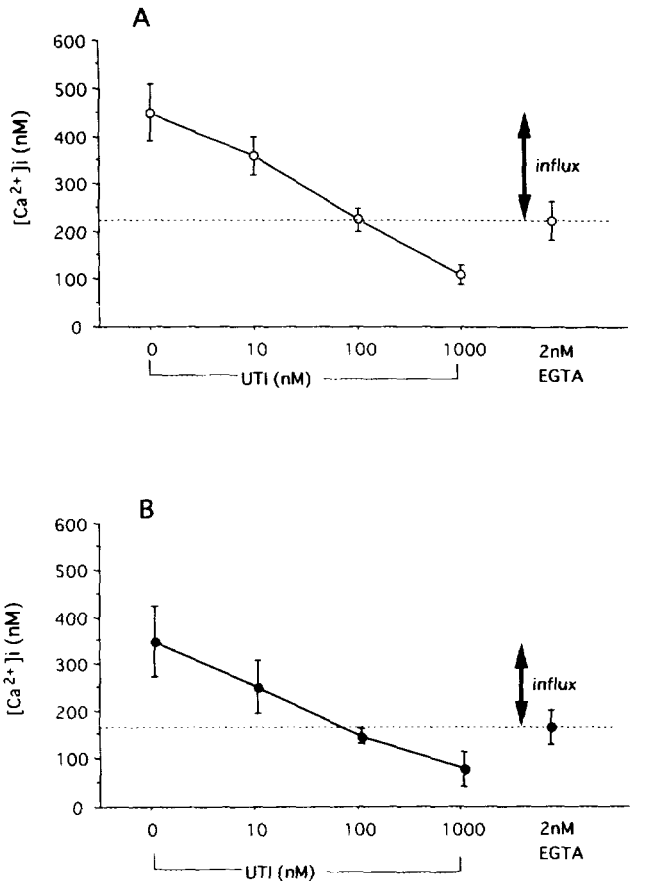

Fig. 2. Dose-dependence of effect of UTI on inhibition of increase of $[Ca^{2+}]_i$ in neutrophils (A) and HUVEC cells (B) induced by LPS. $[Ca^{2+}]_i$ was recorded at 60 s. The right bar shows $[Ca^{2+}]_i$ in the presence of EGTA. The dotted line indicates the level of intracellular Ca^{2+} mobilization by LPS. Therefore, values above the dotted line indicate Ca^{2+} influx.

cells is still controversial. It is reported that the regulation of intracellular Ca^{2+} of these cells is related to the receptor operated Ca^{2+} channel or Na^+/Ca^{2+} pump (22-23). Phosphatidic acid plays a role as a Ca^{2+} ionophore (24). IP_3 is relevant to Ca^{2+} influx by a Ca^{2+} induced Ca^{2+} release system (CICR) (25). These reports suggest that activation of phospholipid is important in Ca^{2+} influx into neutrophils and endothelial cells. UTI may complex with CD14 or phosphatidyl-inisitol, resulting in inhibition of siganl transduction of LPS. Further studies are needed on the relationship between CD14 and Ca^{2+} channels.

Peptide 2A, in domain 2, NLPIVRGPCRAFIQL(83-97), and 2A-1 RGPCRAFI, depressed Ca^{2+} influx completely like UTI (Fig. 2 A, B). The 2B peptide RAFIQLWAFDAVKGK (92-106) inhibited the increase of $[Ca^{2+}]_i$ moderately, but the other peptides tested did not inhibit the increase of $[Ca^{2+}]_i$. 2A and 2B also have trypsin inhibiting activity, but 1A and 1B do not. Thus, the Ca^{2+} inhibitory domain is the same as the trypsin inhibitory domain. The variant form of 2A-1, 2A-1v (RGPCAAFI) also could not prevent Ca^{2+} influx. Thus 92-Arg in UTI is an active site of

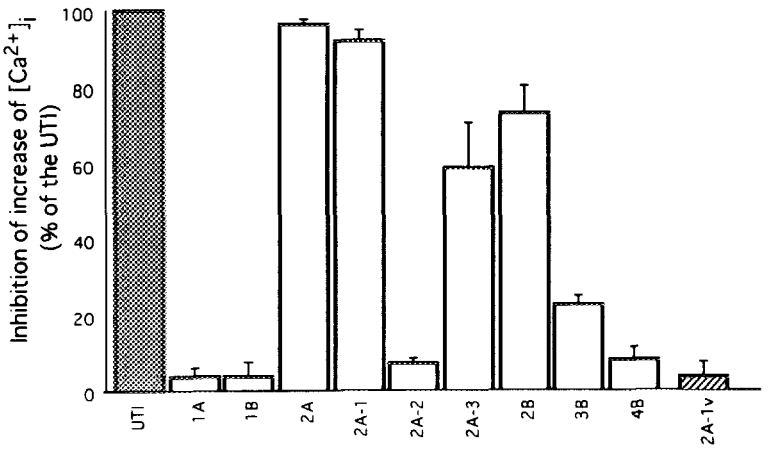

Fig. 3. Effects of UTI peptides on the change of $[Ca^{2+}]_i$ of neutrophils. Inhibitions of calcium influx by each peptide are shown. Note that UTI and peptides **2A** and **2A-1** inhibited Ca^{2+} influx markedly, whereas peptides **2A-3** and **2B** inhibited it moderately.

regulation of Ca^{2+} influx as well as of trypsin inhibition. Ca^{2+} influx was inhibited 55% by 2A-3 (RAF), 65% by 2B (RAFIQLWAFDAVKGK) and 9% by 2A-2 (RGP). Therefore, 2A-3 (RAF) is an essential domain for regulation of Ca^{2+} influx. The difference of amino acid sequences between 2A-2 and 2A-3 is only two amino acids. As AF of 2A-3 is hydropbobic compared to GP of 2A-2, 2A-3 may have high affinity for lipid bilayer, resulting in regulation of Ca^{2+} on cell surface.

The UTI concentrations is increased in cancer tissues (26), inflammation (27) and brain tissue during stress (28). UTI has also been demonstrated in astrocytes in Alzheimer diseases (29). Not only a protease inhibitor but also a physiological calcium channel blocker may act in such pathological conditions. Recently, Calcicludine, a venom peptide of the Kunitz-type protease inhibitor family, was reported to be a potent blocker of high-threshold L-type Ca^{2+} channels with high affinity in cerebellar granule neurons (30). UTI and Calcicludine show high sequence homology. This finding is consistent with our present results suggesting that the Kunitz-type protease inhibitor UTI regulates Ca^{2+} channels in the cell membrane.

We conclude that the Kunitz-type trypsin inhibitor UTI is involved in regulation of the intracellular Ca^{2+} concentration. Its function is ascribed to its trypsin inhibitor region.

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