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Suppression of lipopolysaccharide-induced cytokine production of gingival fibroblasts by a soybean, Kunitz trypsin inhibitor

Hiroshi Kobayashi[1], Ryuji Yoshida[2], Yasufumi Kanada[2], Yoichi Fukuda[3], Tatsuo Yagyu[4], Kiyokazu Inagaki[4], Toshiharu Kondo[5], Noriyuki Kurita[6], Mika Suzuki[1], Naohiro Kanayama[1], Toshihiko Terao[1]

[1]Department of Obstetrics and Gynecology, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka, [2]Food Science Research Institute, Tsukuba R & D Center, Fuji Oil Co. Ltd., Tsukuba-gun, Ibaraki, [3]Hannan R & D Center, Fuji Oil Co. Ltd, Izumisano, Osaka, [4]NetForce Co. Ltd., Nagoya, Aichi, [5]Computer Technology Integration (CTI) Co. Ltd, Nagoya, Aichi, [6]Department of Knowledge-Based Information Engineering, Toyohashi University of Technology, Tempaku-cho, Toyohashi, Japan

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Background: Human bikunin, a Kunitz-type trypsin inhibitor, inhibits inflammation by down-regulating the expression of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) in tumor cells and inflammatory cells.

Objectives: We analyzed the effect of a soybean-derived Kunitz trypsin inhibitor (KTI) on TNF-α production in human gingival fibroblasts stimulated by lipopolysaccharide (LPS), an inflammatory inducer.

Material and methods: Mitogen-activated protein kinase (MAPK) activation and cytokine levels were monitored using western blot and a specific enzyme-linked immunosorbent assay (ELISA).

Results: Here, we show (i) a soybean KTI abrogates LPS-induced up-regulation of TNF-α mRNA and protein expression in a dose-dependent manner in gingival fibroblasts, (ii) KTI also blocks the induction of TNF-α target molecules interleukin-1β (IL-1β) and IL-6 proteins, (iii) inhibition by KTI of TNF-α induction correlates with the suppressive capacity of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 signaling pathways, implicating repressed ERK1/2 and p38 signalings in the inhibition, and (iv) pretreatment of cells with KTI blocked LPS-induced nuclear factor κB (NFκB) activation.

Conclusion: Our results indicate that KTI inhibits LPS-induced up-regulation of cytokine expression possibly through suppression of ERK1/2 and p38 kinase-mediated NFκB activation. These findings may identify anti-inflammatory properties of KTI at the level of gingival fibroblasts and may be relevant to the use of KTI in modulating inflammation, including periodontal disease.

Hiroshi Kobayashi, MD, PhD, Department of Obstetrics and Gynecology, Hamamatsu University School of Medicine, Handayama 1-20-1, Hamamatsu, Shizuoka, 431–3192, Japan
Tel: +81 53 435 2309
Fax: +81 53 435 2308
e-mail: hirokoba@hama-med.ac.jp

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Lipopolysaccharide (LPS) is beneficial to the host by activating the innate immune system, or harmful by inducing inflammation, disseminated intravascular coagulation, multiple organ failure, shock and often death. Inflammatory stimuli induce cytokines, which mediate tissue responses in different phases of inflammation in a sequential and concerted manner (1). Tumor necrosis factor-alpha (TNF-α) is a pleiotropic cytokine secreted by different cell types including macrophages, mastocytes, T and B lymphocytes, and natural killer cells in response to various stimuli, including LPS (2). TNF-α has been identified as a major mediator of inflammatory processes, one of the most dramatic being Gram-negative endotoxic shock (2). We recently demonstrated that LPS induces TNF-α expression possibly through transient phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 signals in isolated human peripheral mononuclear cells (3).

Periodontal disease is the major cause of adult tooth loss and is commonly characterized by a chronic inflammation caused by infection with oral bacteria (4). Blockade of interleukin-1 (IL-1) and TNF may facilitate periodontal wound healing (5). Therefore, such blockade may suppress proinflammatory responses in periodontal disease.

Soybean proteins are widely used in human foods in a variety of forms, including infant formulas, flour, protein concentrates, protein isolates, soy sauces, textured soy fibers, and tofu (6). Protein protease inhibitors are widely distributed in plant seeds (6). The trypsin-chymotrypsin inhibitors from soybeans and from chickpeas inhibit insect midgut proteases, supporting the hypothesis that protease inhibitors comprise a built-in defense mechanism of the seed against insects. Soybean contains the Kunitz trypsin inhibitor (KTI). We showed that KTI could inhibit the up-regulation of urokinase expression through suppression of the mitogen-activated protein kinase (MAPK)-dependent signaling (7). From these observations it may be speculated that KTI induces suppression of proinflammatory responses through suppression of some signaling cascades.

These findings raise the question of whether the induction of TNF-α during inflammation in periodontal disease serves as a target of anti-inflammation by KTI. In this study, we tested this hypothesis by examining the signal transduction mechanism of KTI on the induction of TNF-α by LPS derived from *Escherichia coli* and *Porphyromonas gingivalis* in human gingival fibroblasts. Gingival fibroblasts produce several proinflammatory cytokines in response to direct and indirect stimulations with LPS from the major periodontopathic bacterium, *P. gingivalis*. Our data reveal that KTI blocks LPS-induced up-regulation of TNF-α mRNA and protein expression through suppression of ERK1/2 and p38-mediated signal pathways. To our knowledge, this is the first report of inhibition of LPS-induced TNF-α production by KTI in gingival fibroblasts. Our findings provide new insights into a mechanism of protection against periodontal diseases by KTI.

Material and methods

Reagents

All experiments were performed with LPS from *Escherichia coli* serotype O111:B4 (Sigma Chemical Co., St. Louis, MO, USA). Key experiments were repeated with *P. gingivalis* LPS. For the generation of stock solutions, all reagents were dissolved in endotoxin-free water (Sigma). RPMI 1640 medium, Dulbecco's modified Eagle's medium and fetal calf serum were obtained from Gibco BRL (Rockville, MD, USA). Mouse anti-phospho-Thr^{202}/Tyr^{204} ERK1/2 (specifically detects endogenous levels of ERK1/2, p42/p44, only when phosphorylated at Thr^{202}/Tyr^{204}) and mouse anti-ERK antibodies were purchased from New England Biolabs Inc. (Beverly, MA, USA). Rabbit anti-p38 kinase, rabbit anti-phospho-Tyr^{182} p38 kinase, rabbit anti-JNK2, and rabbit anti-phospho-Thr^{183}/Tyr^{185} JNK antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PD98059, SB203580 and SP600125 were supplied by Calbiochem (La Jolla, CA, USA). The inhibitors were dissolved in dimethyl sulfoxide and used in the following concentrations: PD98059 (10 μM, 30 min; an inhibitor of the ERK pathway), SB203580 (15 μM, 30 min; an inhibitor of p38 MAPK), and SP600125 (50 μM, 30 min; an inhibitor of JNK pathway). A purified soybean KTI was obtained from Fuji Oil Co. Ltd (Osaka, Japan).

Preparation of *Porphyromonas gingivalis* lipopolysaccharides

Porphyromonas gingivalis ATCC 33277 (wild type) were grown anaerobically (10% CO_2, 10% H_2, and 80% N_2) in enriched brain heart infusion medium and on enriched tryptic soy agar (8). *Porphyromonas gingivalis* cells grown in enriched brain heart infusion medium for 48 h were harvested by centrifugation at 9000 *g* for 5 min, washed with phosphate-buffered saline (pH 7.4). *Porphyromonas gingivalis* LPS was purified by the cold $MgCl_2$/ethanol procedure (9). *Porphyromonas gingivalis* LPS preparations were suspended in LPS-free water.

Purification and cell culture

Human gingival fibroblasts were prepared from the explants of normal gingival tissues of 20-year-old and 28-year-old female patients (10). Informed consent was obtained prior to inclusion in the study. The explants were cut into pieces and cultured in 100-mm diameter tissue culture dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum with a medium change every 3 days for about 14 days until confluent cell monolayers were formed. After three to four subcultures by trypsinization, homogeneous, slim, spindle-shaped cells grown in characteristic swirls were obtained. The cells were used as confluent monolayers at subculture levels 7 through 15. Cells were plated at 1×10^6 cells/ml in Dulbecco's modified Eagle's medium plus 10%

fetal calf serum media and were plated in Costar (Corning Inc., Corning, NY, USA) 6-well culture dishes. Cells were stimulated with LPS and incubated at 37°C for specific lengths of time (up to 24 h, TNF-α; 6 and 24 h, IL-1β and IL-6). At the end of the incubation, supernatants were removed and assayed for cytokines. Gingival fibroblasts prepared from two young females were assayed separately.

Stimulation protocol

To analyze the inhibitory effect of KTI on TNF-α release *in vitro*, gingival fibroblasts (1×10^6 cells/well) were incubated with KTI (5 μM), and 2 h later the cells were stimulated with *E. coli* LPS (10 ng/ml) or *P. gingivalis* LPS (30 ng/ml) alone or in combination with pharmacological inhibitors for specific lengths of time (37°C, 5% CO_2). In experiments to determine the effects of MEK, JNK, and p38 inhibitors, each compound was added at various concentrations 30 min before addition of LPS. Supernatants were harvested and stored at −20°C until the TNF-α content was measured by enzyme-linked immunosorbent assay (ELISA).

Determination of cytokines

Human TNF-α was monitored using the TNF-α ELISA from CosmoBio (Tokyo, Japan). The assays were performed as described by the manufacturer. The quantitative analyses of IL-1β and IL-6 were performed by a specific ELISA (CosmoBio). Culture supernatants were used at a dilution of 1:2–1:100 and measured twice. The sensitivities were less than 16, 4 and 8 pg/ml for TNF-α, IL-1β and IL-6, respectively. The intra-assay variations of these assays were < 10%.

MTT assay

To measure cell viability, the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was performed. The cultured gingival fibroblast cells (5×10^5/well) were incubated with MTT (420 μg/ml, Sigma), which is metabolized by living cells in 3 h. MTT crystals were solubilized with HCl-isopropanol (150 μl/well). MTT is cleaved by living cells to yield a dark blue formazan product. Plates were analyzed in an ELISA plate reader at 570 nm with a reference wavelength of 690 nm.

Western blot analysis

The cells treated with or without various agents for indicated times were washed with phosphate-buffered saline. Then 1×10^6 cells were lysed in 750 μl of lysis buffer at 4°C for 15 min and scraped with a rubber policeman. The protein concentrations in the supernatants of cell extracts were measured by the Bio-Rad (Hercules, CA, USA) protein assay. All samples were stored at −70°C until use. In parallel, cells treated in the same condition in different dishes were harvested and counted using a hemocytometer. Centrifuged lysates (50 μg) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane by semi-dry transfer (11). Membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween 20 and 2% bocine serum albumin. Blots were probed with the following primary antibodies overnight at 4°C: phospho-ERK1/2, ERK1/2, phospho-p38 MAPK, p38 MAPK, phospho-JNK, and JNK were detected by specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The immunoblots were visualized by chemiluminescence with the ECL kit from Amersham Biosciences.

Reverse transcription–polymerase chain reaction

RT–PCR was performed as described (12). Total RNA was isolated from gingival fibroblasts using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). TNF-α primers were: forward, 5′-CAGAGGGAAGAGTCCCCCAG-3′; reverse, 5′-CCTTGGTCTGGTAGGAGACG-3′. Human β-actin primers (positive control) were: forward, 5′-ATGTTTGAGACCTTCAACAC-3′; reverse, 5′-CAGGTCACACTTCATGATGC-3′.

Statistics

Data are expressed as mean ± SD of three independent triplicate experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Student's *t*-test. $p < 0.05$ was considered statistically significant.

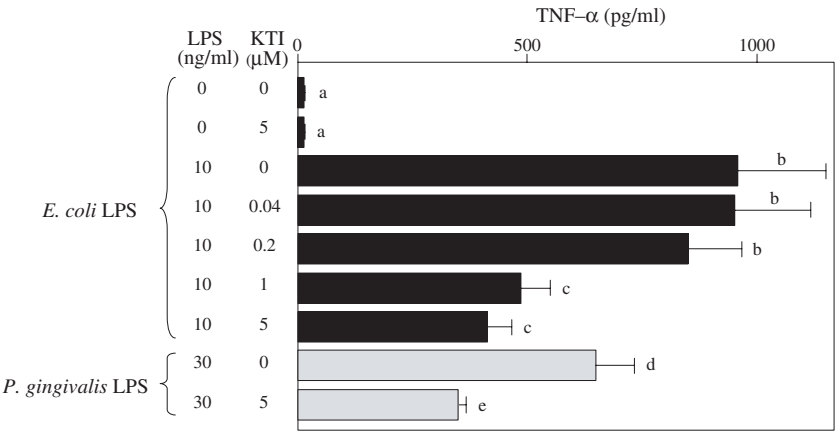

Fig. 1. Inhibition of tumor necrosis factor-α (TNF-α) induction by Kunitz trypsin inhibitor (KTI). Gingival fibroblasts were treated with or without KTI at the indicated concentrations for 2 h, followed by *Escherichia coli* lipopolysaccharide (LPS) (10 ng/ml) or *Porphyromonas gingivalis* LPS (30 ng/ml) stimulation for 12 h. The cell culture media were harvested to measure TNF-α production. Results are the mean ± SD of four different determinations; unlike letters (a–e) represent statistical differences ($p < 0.05$).

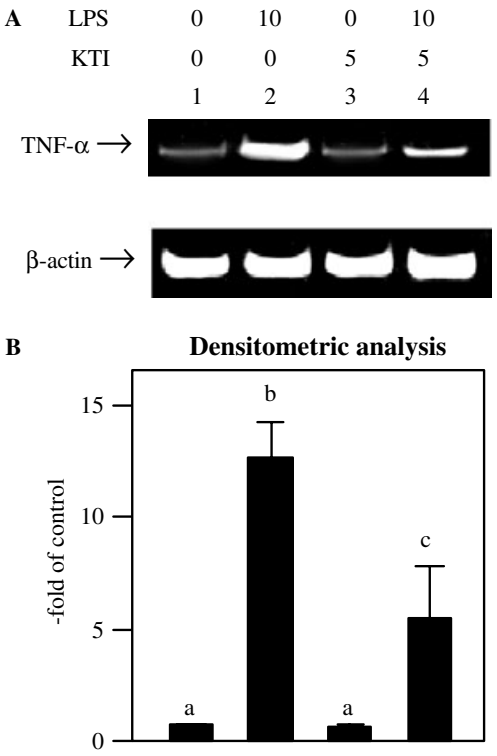

Fig. 2. Kunitz trypsin inhibitor (KTI) prevents tumor necrosis factor-α (TNF-α) gene expression. (A) Gingival fibroblasts were pretreated with KTI (5 μM) or medium for 2 h and subsequently challenged with lipopolysaccharide (LPS: 10 ng/ml) for 6 h. (B) Blots were scanned and analyzed for quantification with the Macintosh software. Band intensities for TNF-α were normalized to the corresponding band intensities for β-actin. Shown are mean mRNA levels calibrated to the amount of β-actin mRNA as determined by real-time polymerase chain reaction ± SD ($n = 4$). Experiments were repeated four times with essentially identical results. Data from four experiments were averaged and are represented as the mean ± SD, expressed as fold increase with respect to non-stimulated cells (lane 1, control). Unlike letters (a–c) represent statistical differences ($p < 0.05$).

Results

Inhibition of lipopolysaccharide-induced up-regulation of tumor necrosis factor-α protein production by Kunitz trypsin inhibitor in gingival fibroblasts

All experiments initially used commercially available *E. coli* LPS. We examined the effect of KTI on LPS-induced production of TNF-α in gingival fibroblasts. A very low amount of TNF-α protein was detected by a specific ELISA for TNF-α in controls (Fig. 1). KTI alone does not affect its production. *Escherichia coli* LPS produces large quantities of TNF-α, reaching a maximum of ~40-fold at 12 h. LPS-induced TNF-α production in gingival fibroblasts was inhibited by pretreatment with KTI in a dose-dependent manner, reaching 55% inhibition at the highest doses of KTI tested (5 μM). The IC_{50} value of the inhibition by KTI is ~1 μM. Thus, KTI significantly blocks LPS-induced production of TNF-α protein in gingival fibroblasts. KTI did not affect MTT activity (data not shown). Furthermore, when gingival fibroblasts were treated with KTI, they constitutively expressed ERK1/2, JNK and p38 MAPK proteins (see Fig. 2). These data demonstrate that KTI does not cause marked damage to the cells at the concentrations tested and thus it does not exhibit a generalized reduction in protein synthesis or function.

In a separate experiment, we confirmed that KTI also reduced *P. gingivalis* LPS-induced up-regulation of TNF-α (see end of the Results section).

Inhibition of lipopolysaccharide-induced up-regulation of tumor necrosis factor-α mRNA expression by Kunitz trypsin inhibitor in gingival fibroblasts

Gingival fibroblasts were pretreated with 5 μM KTI or medium for 2 h and subsequently challenged with 10 ng/ml *E. coli* LPS for 6 h. RNA was isolated from gingival fibroblasts and the level of TNF-α mRNA was determined by quantitative RT–PCR (Fig. 2). Incubation of gingival fibroblasts with LPS led to a 12-fold increase in TNF-α mRNA levels. Pretreatment with KTI abolished TNF-α mRNA levels by 60% in response to LPS. KTI alone did not decrease TNF-α mRNA levels.

Kunitz trypsin inhibitor inhibits lipopolysaccharide-induced phosphorylation of extracellular signal-regulated kinase 1/2 and p38

Figure 3 shows the effects of KTI on the phosphorylation of ERK1/2 (Fig. 3A), JNK (Fig. 3B) and p38 (Fig. 3C) after exposure to 10 ng/ml LPS. Gingival fibroblasts were pretreated for 2 h with KTI or for 30 min with each pharmacological inhibitor before LPS exposure. LPS-induced phosphorylation of ERK1/2 (Fig. 3A, lane 3) and p38 (Fig. 3C, lane 3) was significantly inhibited by 5.0 μM KTI. On the other hand, LPS-induced phosphorylation of JNK (Fig. 3B, lane 3) was not inhibited by KTI (5 μM). In a parallel experiment, pretreatment with 10 μM PD98059 (MEK), 50 μM SP600125 (JNK) and 15 μM SB203580 (p38) had a marked effect on LPS-induced ERK1/2, JNK and p38 phosphorylation, respectively.

Figure 4 showed that pretreatment with 10 μM PD98059, 50 μM SP600125 and 15 μM SB203580 had a significant effect on LPS-induced TNF-α protein expression in gingival fibroblasts, demonstrating that LPS-induced TNF-α protein expression is mediated at least by activation of the ERK1/2,

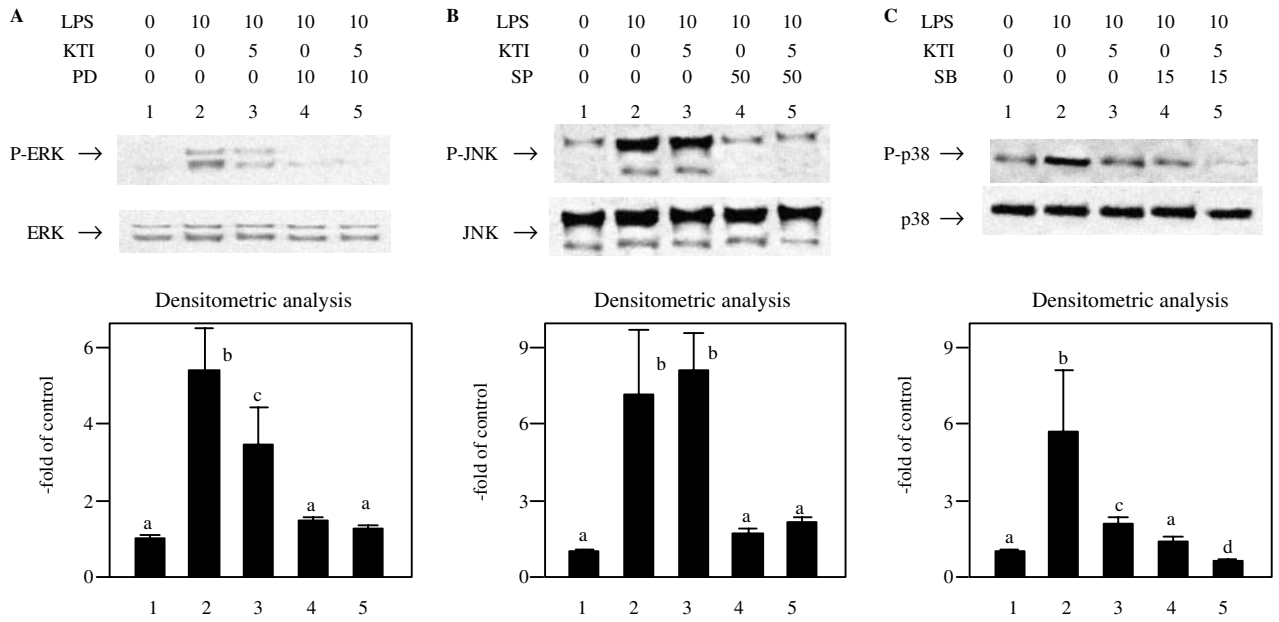

Fig. 3. Inhibition by Kunitz trypsin inhibitor (KTI) on lipopolysaccharide (LPS)-induced phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38. Human gingival fibroblasts pretreated with or without KTI (5 μM; lanes 3 and 5) and pharmacological inhibitors [PD98059 (PD), SP600125 (SP), or SB203580 (SB); lanes 4 and 5] were stimulated with LPS (10 ng/ml; lanes 2–5) for 15 min. Cells were lysed with lysis buffer, and the protein content of each sample was quantitated. Each sample (50 μg) was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and probed with anti-phospho-ERK1/2 (A, upper panel), anti-phospho-JNK (B, upper panel), or anti-phospho-p38 (C, upper panel) to detect the phosphorylated ERK1/2, JNK, or p38 proteins. Blots were stripped and reprobed with anti-ERK (A, lower panel), anti-JNK (B, lower panel), or anti-p38 (C, lower panel), respectively. Experiments were repeated three times with essentially identical results. Blots were scanned and analyzed for quantification with the Macintosh software. Band intensities for phosphorylated proteins were normalized to the corresponding band intensities for total proteins. Data from three experiments were averaged and are represented as the mean ± SD, expressed as fold increase with respect to lane 1. Unlike letters (a–d) represent statistical differences ($p < 0.05$).

JNK and p38 signaling pathways. Furthermore, we tested whether addition of each pharmacological inhibitor to the LPS-stimulated gingival fibroblasts pretreated with KTI significantly suppresses TNF-α expression. The addition of PD98059 or SB203580 together masked the KTI's action. These results allow us to speculate that KTI inhibits LPS-induced TNF-α expression predominantly through suppression of ERK1/2 and p38 signaling pathways, but KTI does not completely suppress these signalings.

Kunitz trypsin inhibitor inhibits lipopolysaccharide-induced nuclear factor κB activation via extracellular signal-regulated kinase 1/2 and p38 kinase signalings

As described previously (12), LPS treatment caused activation of nuclear factor κB (NFκB) as demonstrated by the measures of IκB (inhibitor of NFκB) phosphorylation (Fig. 5). NFκB activation was determined by examining phosphorylation of IκB-α because degradation of IκB-α via its phosphorylation is necessary for nuclear translocation of NFκB and subsequent activation of target gene expression. Therefore, the level of the IκB-α protein decreased as the phosphorylation level of the IκB-α protein increased (lane 1 vs. lane 2). Pretreatment of KTI (5 μM) (lane 3), direct inhibition of ERK1/2 with PD98059 (50 μM, lane 4), or direct inhibition of p38 kinase with SB203580 (15 μM, lane 6) blocked LPS-induced IκB-α phosphorylation and LPS-induced IκB-α degradation. However, SP600125 (50 μM, lane 5) did not inhibit phosphorylation of IκB-α. Taken together, these data suggest that inhibition of LPS-induced ERK1/2 and p38 kinase activation by KTI is responsible for the inhibition of NFκB activation.

Inhibition of tumor necrosis factor-α target cytokines by Kunitz trypsin inhibitor

TNF-α mediates the production of many other cytokines during inflammation (13), in particular the production of IL-1β and IL-6. We tested whether suppression of TNF-α production by KTI has an effect on the production of TNF-α target molecules in gingival fibroblasts. As expected, Fig. 6 shows that LPS induces large increases in the production of IL-1β and IL-6 proteins. KTI markedly blocks LPS-induced production of IL-6 and IL-1β at both the 6 h (Fig. 6A) and 24 h (Fig. 6B) time points.

Assessment of generality of the results from *Escherichia coli* lipopolysaccharide

All experiments used *E. coli* LPS. As *E. coli* has not been associated with

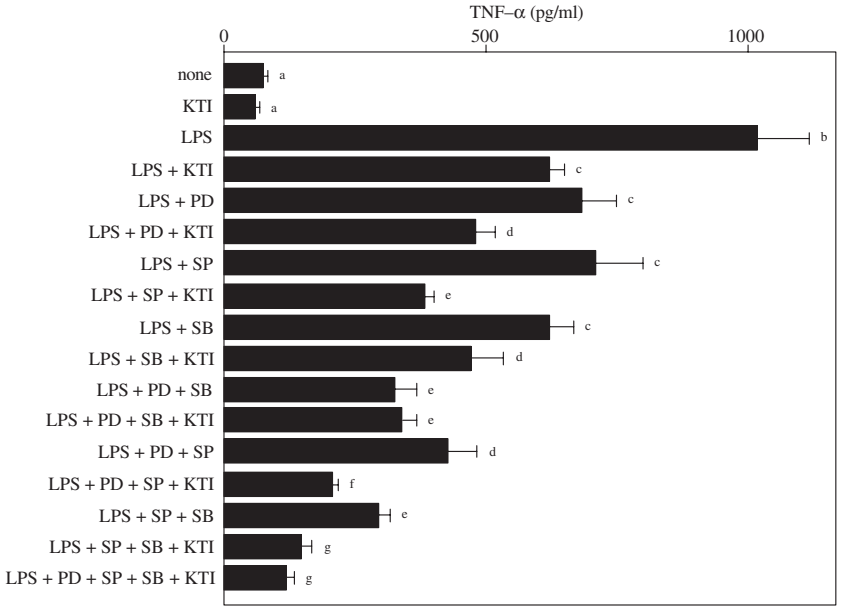

Fig. 4. Inhibition of tumor necrosis factor-α (TNF-α) induction by Kunitz trypsin inhibitor (KTI) and/or pharmacological inhibitors. Gingival fibroblasts were treated with or without KTI (5 μM) and/or pharmacological inhibitors at the indicated concentrations (see Fig. 3), followed by *E. coli* lipopolysaccharide (LPS) (10 ng/ml) stimulation for 12 h. The cell culture media were harvested to measure TNF-α production. The means and SD of four treatments are presented. Unlike letters (a–g) represent statistical differences ($p < 0.05$). PD, PD98059; SP, SP600125, or SB, SB203580.

periodontal disease, the inhibition experiments were repeated using LPS from a periodontal disease-associated organism, *P. gingivalis*. Key experiments were repeated with *P. gingivalis* LPS. As shown in Fig. 1, consistent with our original findings, *P. gingivalis* LPS (30 ng/ml) treatment of cells results in the induction of TNF-α protein. 5 μM KTI reduced *P. gingivalis* LPS-induced up-regulation of TNF-α by ~50%.

Discussion

Efforts to understand the pathogenesis of periodontal diseases have been underway for decades (14). Recent anti-cytokine therapy for inflammatory diseases aimed to inhibit cytokine function. These data allow us to speculate that interference with inflammatory principles expressed in the periodontal tissue may constitute novel therapeutic opportunities for the prevention, amelioration, or treatment of periodontal diseases.

Analyses of the molecular mechanism by which a soybean KTI suppresses activation of target proteins may provide new insights into a mechanism of anti-inflammatory action. We have been examining the mechanism of KTI's action (7). Our previous experiments demonstrated that KTI has not only an anti-trypsin activity but can also block up-regulation of urokinase expression through a mechanism that involves the inhibition of activity of the ERK1/2 in tumor cells (7). In the present study, we demonstrated that preincubation with KTI (5 μM, 2 h) suppresses the proinflammatory reaction to LPS treatment, as demonstrated by suppressed production of TNF-α mRNA and protein possibly through suppression of NFkB activation in gingival fibroblasts. Pre-exposure to KTI also suppresses LPS-induced IL-1β and IL-6 formation. The effect of KTI on the gingival fibroblasts response to LPS lasted for at least 24 h and was already fully established within 6 h of incubation. Our results indicate that KTI blocked LPS-induced ERK1/2 and p38 kinase, which results in suppression of NFκB-induced TNF-α expression. It is likely that KTI suppresses NFkB nuclear translocation, IkB degradation, and NFkB-inducing kinase (NIK)-induced NFkB transcriptional activation. These results suggest that the molecular target of the effects of KTI may be the ERK1/2 and p38-dependent NFκB activation cascade. At this time, we have no data that the KTI's target is IkB phosphorylation by IkB kinase (IKK), whose activation follows NIK activation and precedes IkB degradation in the NFkB pathway. The interaction of KTI with the signaling molecules can be either direct, in which it directly modulates target signalings, or indirect, in which it influences the functional groups of the protein by affecting the environment in cells.

Induction by LPS is mediated through complex signal transduction pathways involving both transcriptional (15, 16) and post-transcriptional mechanisms (2). Macrophage activation by LPS results in NFkB-dependent activation of TNF-α gene transcription, derepression of TNF-α mRNA translation, and secretion of TNF-α protein (17). It is unclear at present that the activation of the ERK1/2 and p38 signaling pathway by LPS is sufficient for the induction of TNF-α expression in gingival fibroblasts, although ERK1/2 and p38 pathway plays a major role in the inhibition of TNF-α function by KTI. In macrophages, KTI suppressed LPS-induced up-regulation of cytokine expression through suppression of phosphorylation of three MAPK pathways, ERK1/2, JNK and p38 (unpublished data). Thus, KTI can repress JNK activation in macrophages, but not in gingival fibroblasts. The molecular mechanisms of KTI-induced JNK-dependent signaling cascade are still unknown in gingival fibroblasts.

In this study, we used a cell population from biased subjects (young females). Therefore, we must take into consideration that there are age- and gender-dependent changes in gene expression and cell function. Notwithstanding these limitations, these findings allow us to speculate that a soybean KTI may exhibit anti-inflammatory activity and may be relevant to the use of KTI in modulating inflam-

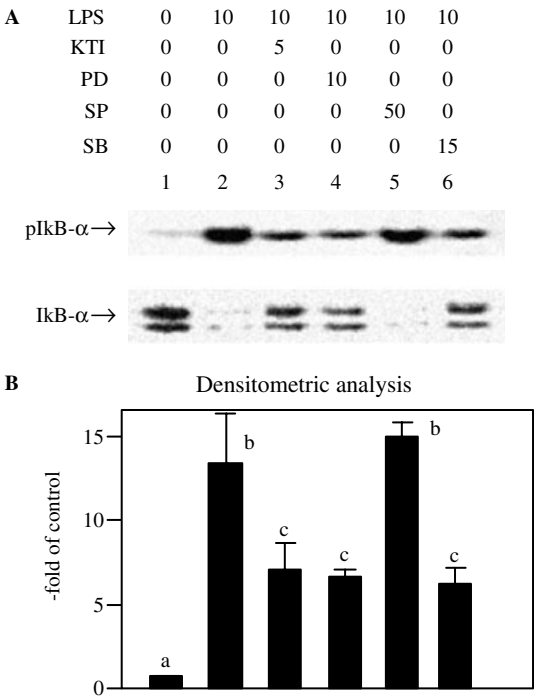

Fig. 5. Kunitz trypsin inhibitor (KTI) blocks lipopolysaccharide (LPS)-induced nuclear factor κB (NFκB) activation. (A) Gingival fibroblasts were pretreated with KTI (5 μM, lane 3) for 2 h or PD98059 (PD: 10 μM, lane 4), SP600125 (SP: 50 μM, lane 5), or SB203580 (SB: 15 μM, lane 6) for 30 min and then stimulated with LPS (10 ng/ml) for 1 h. Expression levels of IκB-α and phosphorylated IκB-α (pIκB-α) were determined by western blot analysis. (B) Blots of IκB-α in (A) were scanned and the band intensities were quantitated. The band intensity values were used to determine the relative amount of pIκB-α. Results are the mean ± SD of four different determinations; unlike superscripts (a–c) are different ($p < 0.05$).

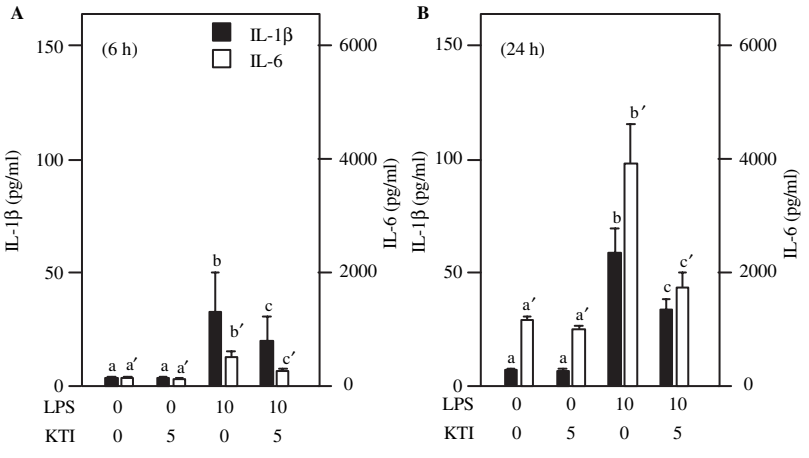

Fig. 6. Inhibition of lipopolysaccharide (LPS)-induced interleukin-1β (IL-1β) and IL-6 protein production by Kunitz trypsin inhibitor (KTI). Gingival fibroblasts treated with or without KTI for 2 h, followed by stimulation with LPS (10 ng/ml) for 6 h (A) and 24 h (B), respectively. The culture medium was collected and assayed for IL-1β (solid bar) or IL-6 (open bar) using enzyme-linked immunosorbent assay (ELISA) kits specific for IL-1β or IL-6, respectively. The results represent the means + SD of four treatments. Unlike letters (a–c and a′–c′) represent statistical differences ($p < 0.05$).

mation-mediated diseases, including periodontal disease. We have been examining a role of KTI as an anti-inflammatory agent in an *in vivo* animal model.

In conclusion, this study is the first report of inhibition of *E. coli* and *P. gingivalis* LPS-induced TNF-α production by KTI in human gingival fibroblasts. Our results suggest a mechanism of anti-inflammation by KTI through control of cytokine induction during inflammation, possibly through suppression of ERK1/2 and p38-mediated NFkB activation. This work will lay the foundation for future studies directed towards the development of prevention or treatment modalities for gingival infection based on blocking the overexpression of cytokines at the cellular level.

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