Bikunin Inhibits Lipopolysaccharide-Induced Tumor Necrosis Factor Alpha Induction in Macrophages

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Bikunin, a Kunitz-type protease inhibitor, exhibits anti-inflammatory activity in protection against cancer and inflammation. To investigate the molecular mechanism of this inhibition, we analyzed the effect of bikunin on tumor necrosis factor alpha (TNF-α) production in human peripheral mononuclear cells stimulated by lipopolysaccharide (LPS), an inflammatory inducer. Here, we show the following results. (i) LPS induced TNF-α expression in time- and dose-dependent manners through phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase pathways. (ii) Bikunin inhibits LPS-induced up-regulation of TNF-α protein expression in a dose-dependent manner, reaching 60% inhibition at the highest doses of bikunin tested (5.0 μM). (iii) Inhibition by bikunin of TNF-α induction correlates with the suppressive capacity of ERK1/2, JNK, and p38 signaling pathways, implicating repressions of at least three different signals in the inhibition. (iv) Bikunin blocks the induction of TNF-α target molecules interleukin-1β (IL-1β) and IL-6 proteins. (v) Bikunin is functional in vivo, and this glycoprotein blocks systemic TNF-α release in mice challenged with LPS. (vi) Finally, bikunin can prevent LPS-induced lethality. In conclusion, bikunin significantly inhibits LPS-induced TNF-α production, suggesting a mechanism of anti-inflammation by bikunin through control of cytokine induction during inflammation. Bikunin might be a candidate for the treatment of inflammation, including septic shock.

The importance of inflammation in the pathological responses to cancer or to endotoxins of foreign origin is well recognized. Inflammatory stimuli induce cytokines, which mediate tissue responses in different phases of inflammation in a sequential and concerted manner (19). Regulation of cytokine induction serves as a key mechanism of inflammation control by endogenous or exogenous chemicals.

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 lipopolysaccharide (LPS) (3). TNF-α has been identified as a major mediator of inflammatory processes, one of the most dramatic being gram-negative endotoxin shock (3). This cytokine mediates early-stage responses of inflammation by regulating the production of other cytokines, including interleukin-1β (IL-1β) and IL-6. Abnormalities in the production or function of TNF-α play essential roles in many inflammatory lesions (7, 21, 22).

Bikunin, a Kunitz-type protease inhibitor found in human amniotic fluid and urine, exhibits anti-inflammatory and anti-metastatic functions in animals (13) and humans (15). The clinical efficacy of bikunin therapy has also been investigated in patients with acute pancreatitis, lung injury (26), severe sepsis (2), preterm delivery (9), Stevens-Johnson syndrome, toxic epidermal necrolysis (11), and advanced cancers (15), as well as for prevention of surgical stress (30). There are several reports suggesting that bikunin is a useful and effective therapy for controlling each of these diseases without any side effects (2, 9, 11, 15, 26, 30).

The broad spectrum of the biological functions of bikunin suggests the existence of multiple molecular targets that mediate diverse responses to the compounds in cells (33). Identifying the target molecules can facilitate the design of better therapeutic agents for protection against certain conditions associated with cancer and inflammatory diseases. Current understanding of the mechanism of action by bikunin comes mostly from studies on the suppression of several signaling cascades (32). Upon exposure to bikunin, CD44 associates with an as-yet-identified receptor for bikunin and modulates the transcription of target genes through mitogen-activated protein kinase (MAPK)- and phosphoinositide-3-kinase-dependent transcription (32). Thus, suppression of signaling activation can account for bikunin’s preventive action against cancer. However, such mechanisms do not readily explain the anti-inflammatory function of bikunin in humans, which is largely unaddressed at present.

The pivotal role of TNF-α in inflammation and the anti-inflammatory activity of bikunin raise the question of whether the induction of TNF-α during inflammation serves as a target of anti-inflammation by bikunin. In this study, we tested this hypothesis by examining the effect of bikunin on the induction of TNF-α by LPS in human peripheral mononuclear cells.
(HPMC). Our data reveal that bikunin blocks LPS-induced expression of TNF-α in both a time- and dose-dependent manner in HPMC. To our knowledge, this is the first report of inhibition of LPS-induced TNF-α production by bikunin in HPMC. Our findings provide new insights into the mechanism of protection against inflammatory diseases by bikunin.

MATERIALS AND METHODS

Reagents. All experiments were performed with LPS from Salmonella enteritidis (Sigma). 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 Invitrogen Japan K.K. (Tokyo, Japan). Mouse anti-phospho-Thr202/Tyr204 extracellular signal-regulated kinases 1 and 2 (ERK1/2) and mouse anti-ERK antibodies were purchased from New England Biolabs, Inc. (Beverly, Mass.). PD98059, SB203580, and SP600125 were supplied by Calbiochem (La Jolla, Calif.). PD98059, SB203580, and SP600125 are MEK1/2-specific, p38 kinase-specific, and c-Jun N-terminal kinase (JNK)-specific inhibitors, respectively. 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 the JNK pathway). Rabbit anti-p38 kinase, rabbit anti-phospho-Thr202/Tyr204 p38 kinase, rabbit anti-JNK2, and rabbit anti-phospho-Thr183/Tyr185 JNK antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.).

Monocyte purification and cell culture. HPMC were isolated by a combination of Ficoll-Hypaque gradient centrifugation (25). This procedure resulted in populations of monocytes of greater than 90% purity as determined by Wright-Giemsa staining. Cells were plated at 2 × 10⁶ cells per ml in Dulbecco’s modified Eagle’s medium or RPMI 1640 plus 10% fetal calf serum medium. Cells were plated in Costar (Cambridge, Mass.) 12-well culture dishes with 1 ml of cell suspension per well. 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). In experiments to determine the effects of MEK, JNK, and p38 inhibitors, each compound was added at various concentrations 5 min before the addition of LPS. At the end of the incubation, supernatants were removed and assayed for cytokines.

Stimulation protocol. To analyze the inhibitory effect of bikunin on TNF-α release in vitro, HPMC (2 × 10⁶ cells/well) were incubated with bikunin (1 μM), and 60 min later the cells were stimulated with LPS (10 ng/ml alone or in combination with pharmacological inhibitors for 12 h (37°C; 5% CO2). At this time point, supernatants were harvested and stored at −20°C until the TNF-α content was measured by enzyme-linked immunosorbent assay (ELISA).

Mice and injection protocol. C57BL/6 mice were bought from Japan SLC, Hamamatsu, Japan. All mice were ordered at an age of 8 to 9 weeks and housed in our own animal facility. To induce endotoxin shock, mice were intraperitoneally (i.p.) with D-GalN (D-galactosamine; 4 mg/100 g) were injected with each subline. To our knowledge, this study is the first report of inhibition of LPS-induced TNF-α production by bikunin in HPMC. The log rank test was used to analyze the survival of animals injected with each subline.

FIG. 1. Time course of phosphorylation of ERK1/2, JNK, or p38 in response to LPS stimulation. (A) A representative experiment showing that phosphorylation of ERK1/2 (p-ERK1/2), p-JNK, or p-p38 in HPMC treated with 10 ng of LPS/ml for the indicated times was detected with anti-phospho-ERK1/2, anti-phospho-JNK, or anti-phospho-p38 antibodies, respectively. After stripping phosphor-specific antibodies, total protein levels of ERK1/2, JNK, or p38 in the same samples were detected with anti-ERK1/2, anti-JNK, or anti-p38 antibodies. (B) The phosphorylation level of protein in each group was presented as a ratio of phosphorylated protein to total protein, which was then normalized to each untreated time control. Data represent means ± standard deviations (SD) for three experiments. *P < 0.05 compared with time control. Black column, p-ERK/ERK ratio; white column, p-JNK/JNK ratio; gray column, p-p38/p38 ratio.
RESULTS

The transcriptional and posttranscriptional regulation of TNF-α biosynthesis by LPS in animal macrophage cells is well characterized (34). We initially examined whether LPS induces TNF-α protein expression in isolated HPMC. LPS can induce a dose-dependent TNF-α expression with a 50% effective concentration of 5 ng/ml (data not shown). The response is time dependent; the response reaches a maximum after 12 h of treatment with 10 ng of LPS/ml (data not shown). TNF-α protein expression in LPS-treated HPMC increased 50-fold compared with time controls after 12 h of treatment. The increase in TNF-α production was abolished in the presence of 10 μM cycloheximide, an inhibitor of protein synthesis that was applied 30 min prior to and during the LPS treatment (data not shown).

LPS-induced phosphorylation of ERK1/2, JNK, and p38 in HPMC. It has been reported (29) that ERK, JNK, and p38 are activated to a similar extent (5- to 10-fold) in human monocytes in response to LPS. Maximal kinase activity is observed at 15 to 30 min for all of the pathways. The activation of the ERK pathway is a major component of the effect of LPS (29). Therefore, the regulation of tyrosine phosphorylation of MAP kinase (ERK1/2) by LPS was examined in HPMC. As shown in Fig. 1A, top panel, phosphorylation of ERK1/2 was detected after 5 min of exposure to 10 ng of LPS/ml, reached a maximum at 15 min, and then declined within 30 min. The phosphorylation of ERK1/2 was markedly increased 5.5-, 7.8-, 1.5-, and 1.3-fold in three experiments (P < 0.05 compared with time control) after 5, 15, 30, and 60 min of exposure to LPS, respectively. Total ERK1/2 protein levels remained relatively constant during the 2-h stimulation.

We next characterized the effect of LPS on phosphorylation and activation of JNK and p38. LPS (10 ng/ml) also induced a rapid phosphorylation of JNK (Fig. 1A, middle panel) and p38 (Fig. 1A, bottom panel) in HPMC. The effect on phosphorylated JNK and p38 reached a maximum within 15 min and mostly disappeared within 30 to 120 min. These results suggest that LPS transiently activates ERK1/2, JNK, and p38 signaling in HPMC.

Inhibition of LPS-induced TNF-α production by bikunin in HPMC. To analyze the mechanism of anti-inflammation by bikunin, we examined the effect of this compound on LPS-induced production of TNF-α in HPMC. A very small amount of TNF-α protein was detected by a specific ELISA for TNF-α in controls (Fig. 2). Bikunin alone does not affect the production of TNF-α. Large quantities of TNF-α were produced in response to LPS, reaching a maximum of ~50-fold at 12 h. LPS-induced TNF-α production in HPMC was inhibited by bikunin in a dose-dependent manner, reaching 60% inhibition at the highest doses of bikunin tested (5.0 μM). Thus, bikunin significantly blocks LPS-induced production of TNF-α protein in HPMC. Bikunin did not affect MTT activity, given either alone or in combination (data not shown). Furthermore, when HPMC were treated with bikunin, they constitutively expressed ERK1/2, JNK, and p38 MAPK proteins (Fig. 3). These data demonstrate that bikunin 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.

Next, we analyzed the concentration-response curves. Bikunin inhibits LPS-induced TNF-α production in a dose-dependent manner (Fig. 2). The 50% inhibitory concentration value of the inhibition by bikunin is ~1.0 μM, which is similar to the potency of bikunin for other biological responses, such as the suppression of urokinase plasminogen activator (uPA) expression or cancer invasion (16). Therefore, the inhibition by bikunin is moderate and may be mediated through a mechanism analogous to the suppression of uPA expression by bikunin.

Bikunin inhibits LPS-induced phosphorylation of ERK1/2, JNK, and p38. Figure 3 shows the effects of bikunin on the
phosphorylation of ERK1/2, JNK, and p38 after exposure to 10 ng of LPS/ml. HPMC were pretreated for 60 min with bikunin or for 5 min with each pharmacological inhibitor before LPS exposure. LPS-induced phosphorylation of ERK1/2, JNK, or p38 was inhibited by 1.0 μM bikunin (Fig. 3, lanes 3). In a parallel experiment, pretreatment with 10 μM PD98059 (MEK), 50 μM SP600125 (JNK) and 15 μM SB203580 (p38) had significant effects on LPS-induced ERK1/2, JNK, and p38 phosphorylation, respectively.

Figure 4 shows that pretreatment with 10 μM PD98059, 50 μM SP600125, and 15 μM SB203580 had significant effect on LPS-induced TNF-α protein expression in HPMC, demonstrating that LPS-induced TNF-α protein expression is mediated by activation of the ERK1/2, JNK, and p38 signaling pathways. In addition, bikunin inhibits LPS-induced TNF-α expression, possibly through suppression of not only ERK1/2 signaling but also JNK and p38 pathways.

**Inhibition of TNF-α target cytokines by bikunin.** TNF-α mediates the production of many other cytokines during inflammation (21), in particular, the production of IL-1β and...
We tested whether suppression of TNF-α production by bikunin has an effect on the production of TNF-α target molecules in HPMC. As expected, Fig. 5 shows that LPS induces large increases in the production of IL-1β and IL-6 proteins 24 h after treatment. Bikunin markedly blocks LPS-induced production of IL-1β and IL-6 at both the 6- and 24-h time points (Fig. 5).

**Suppression of TNF-α release in vivo by bikunin.** To examine whether TNF-α was circulating in mice treated with LPS, sera from each mouse were assayed for TNF-α in sandwich ELISAs. TNF-α serum levels were determined 0.5, 1, and 3 h after the LPS challenge. Furthermore, mice were pretreated with bikunin and 60 min later challenged with LPS. Interestingly, the mean concentrations of TNF-α in mice pretreated with bikunin were significantly low (Fig. 6). We found that bikunin is functional in vivo and this glycoprotein blocks systemic TNF-α release in mice challenged with LPS.

**Bikunin protects mice in the endotoxin model.** We finally ascertained the relative survival times of mice after i.p. injection of LPS with or without bikunin (Fig. 7). We therefore used the murine endotoxin–D-GalN shock model as described previously (10). D-GalN sensitizes mice to the toxic effects of TNF-α by several orders of magnitude (12). In this experiment, mice were sensitized with 4 mg of D-GalN/mouse. D-GalN-sensitized C57BL/6 mice were treated with bikunin 60 min before the LPS challenge. The mean survival rate of five mice receiving LPS alone was 20%. In contrast, pretreatment of five mice with bikunin increased the survival rate to 80%. There was a significant increase in the mean survival rate of the group receiving bikunin ($P < 0.05$). These data show that bikunin can...
These findings provide evidence that regulation of TNF-α is promising target for the prevention of inflammatory toxicity. Because TNF-α is the main mediator for a number of inflammatory toxic responses to chemicals, it represents a promising target for the prevention of inflammatory toxicity. These findings provide evidence that regulation of TNF-α production serves as an effective target of anti-inflammation (3).

The mechanism of anti-inflammation by bikunin, which may correlate with antimetastatic activities in cancer (17, 18, 33), is largely unclear at present. In this study, we examined the regulation of TNF-α induction, a key event in inflammatory responses, by bikunin as a mechanism of anti-inflammation. We demonstrated the following. (i) LPS activates the ERK1/2, JNK, and p38 pathways in similar time courses in HPMC. Each specific pharmacological inhibitor fails to completely inhibit JNK, and p38 pathways in similar time courses in HPMC. (ii) HPMC signaling pathways. (v) Most interestingly, bikunin could protect mice in the endotoxin model.

The interaction of bikunin with the signaling molecules can be either direct, in which it binds to the target(s), or indirect, in which it influences the functional groups of the protein by affecting the environment in cells. However, this hypothesis does not exclude other signaling mechanisms in the action of bikunin. Induction by LPS is mediated through complex signal transduction pathways involving both transcriptional (4, 27) and posttranscriptional mechanisms (3). Macrophage activation by LPS results in NF-κB-dependent activation of TNF-α gene transcription, derepression of TNF-α mRNA translation, and secretion of TNF-α protein (24). Analyses of the molecular mechanism by which bikunin suppresses activation of target proteins will provide new insights into the mechanism of anti-inflammatory action by regulation of TNF-α production in future studies.

LPS binds the soluble LPS-binding protein, and the complex binds CD14. CD14 presents the LPS- LPS-binding protein complex to the LPS receptor Toll-like receptor 4 (TLR4) (28). Signals originating in the LPS-triggered TLR4 receptor activate several signaling pathways, which involve ERK1/2. LPS also activates the JNK and p38 MAP kinase pathways, which relieve AU-rich element-dependent posttranscriptional repression, resulting in enhanced TNF-α mRNA stability and translation (24). In addition, LPS activates Tpl2, a serine/threonine kinase type of proto-oncogene, leading to activation of the ERK1/2 pathway, which specifically controls TNF-α induction by regulating nucleocytoplasmic mRNA transport through a mechanism that targets the AU-rich element of the TNF-α mRNA (6, 23). It is unclear at present, however, whether the activation of the ERK1/2, JNK, and p38 signaling pathways by LPS is sufficient for the induction of TNF-α expression in HPMC or which pathway plays a major role in the inhibition of TNF-α function by bikunin.

A previous study provided evidence (31) that bikunin can disrupt dimerization of CD44 proteins in cancer cell lines, which may result in the suppression of receptor-mediated MAP kinase signaling and subsequently reduce uPA at the mRNA and protein levels, indicating that the action of bikunin is not specific for the cytokine pathway. Given the recent recognition that some growth factor receptors can form heterodimers with CD44, we found that bikunin can inhibit these heterodimerizations and inhibit CD44/growth factor-dependent signaling (H. Matsuzaki et al., unpublished data). Bikunin does not alter the ligand binding, whereas it functionally reduces heterodimerization between CD44 and growth factor.

**DISCUSSION**

TNF-α plays a major role in regulating inflammation, mostly through the induction of inflammatory cytokines, including IL-1 (IL-1α and IL-1β), IL-6, IL-8, macrophage inflammatory protein 2, granulocyte-macrophage colony-stimulating factor, and adhesion molecules (1, 21). TNF-α is induced by many external stimuli, the strongest of which is the bacterial endotoxin LPS. Because TNF-α is the main mediator for a number of inflammatory toxic responses to chemicals, it represents a promising target for the prevention of inflammatory toxicity. These findings provide evidence that regulation of TNF-α production serves as an effective target of anti-inflammation (3).

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The interaction of bikunin with the signaling molecules can be either direct, in which it binds to the target(s), or indirect, in which it influences the functional groups of the protein by affecting the environment in cells. However, this hypothesis does not exclude other signaling mechanisms in the action of bikunin. Induction by LPS is mediated through complex signal transduction pathways involving both transcriptional (4, 27) and posttranscriptional mechanisms (3). Macrophage activation by LPS results in NF-κB-dependent activation of TNF-α gene transcription, derepression of TNF-α mRNA translation, and secretion of TNF-α protein (24). Analyses of the molecular mechanism by which bikunin suppresses activation of target proteins will provide new insights into the mechanism of anti-inflammatory action by regulation of TNF-α production in future studies.

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**FIG. 7.** Survival of mice pretreated with or without bikunin after i.p. injection of LPS. D-GalN-sensitized C57BL/6 mice were treated with (five mice) or without bikunin (five mice) 60 min before the LPS challenge. These groups of female mice were monitored for morbidity. Survival of the bikunin-treated mice was significantly longer than that of the control mice (P < 0.05). Experiments 1 and 2 were repeated twice.
cell lines. We have been examining whether bikunin can inhibit agonist-promoted activation of the signaling pathway in cancer substantially reduce receptor-induced tyrosine phosphorylation receptors (31). The disruption of heterodimerization may sub-

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