Gabexate mesilate, a synthetic protease inhibitor, inhibits lipopolysaccharide-induced tumor necrosis factor-α production by inhibiting activation of both nuclear factor-κB and activator protein-1 in human monocytes

Mehtap Yuksel, MD 1,2; Kenji Okajima, MD 1; Mitsuhiro Uchiba, MD 1; Hiroaki Okabe, MD 1

Departments of Laboratory Medicine 1 and Biochemistry 2, Kumamoto University School of Medicine, Kumamoto, Japan
Short title: Inhibition of TNF-α production by gabexate mesilate *in vitro*

Address correspondence to: Kenji Okajima, MD, Department of Laboratory Medicine, Kumamoto University School of Medicine, Honjo 1-1-1, Kumamoto, 860-8556, Japan

Fax: +81-96-373-5281, Phone: +81-96-373-5281

E-mail: whynot@kaiju.medic.kumamoto-u.ac.jp

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Abbreviations: TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; AP-1, activator protein-1; DIC, disseminated intravascular coagulation.

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Abstract

Gabexate mesilate, a synthetic protease inhibitor, was shown to be effective in treating patients with sepsis-associated disseminated intravascular coagulation in which tumor necrosis factor-α (TNF-α) plays a critical role. We demonstrated that gabexate mesilate reduced lipopolysaccharide (LPS)-induced tissue injury by inhibiting TNF-α production in rats. In the present study, we analyzed the mechanism(s) by which gabexate mesilate inhibits LPS-induced TNF-α production in human monocytes in vitro. Gabexate mesilate inhibited the production of TNF-α in monocytes stimulated with LPS. Gabexate mesilate inhibited both the binding of nuclear factor-κB (NF-κB) to target sites and the degradation of IκBα. Gabexate mesilate also inhibited both the binding of activator protein-1 (AP-1) to target sites and the activation of mitogen-activated protein kinase pathways. These observations strongly suggest that gabexate mesilate inhibited LPS-induced TNF-α production in human monocytes by inhibiting activation of both NF-κB and AP-1. Inhibition of TNF-α production by gabexate mesilate might explain at least partly its therapeutic effects in animals given LPS and those in patients with sepsis.
Introduction

On stimulation with lipopolysaccharide (LPS), monocytes release a variety of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (Morrison and Ryan, 1987). TNF-α plays a critical role in the development of disseminated intravascular coagulation (DIC) associated with sepsis (Levi et al., 1999). TNF-α also contributes to activated neutrophil-induced endothelial injury, not only by activating neutrophils (Klebanoff et al., 1986), but also by activating endothelial cells leading to an increase in the expression of endothelial leukocyte adhesion molecules such as E-selectin and intercellular adhesion molecule-1, both of which enable activated neutrophils to adhere to the endothelial cell surface (Mulligan et al., 1991).

Gabexate mesilate is a synthetic serine protease inhibitor that has anticoagulant activities (Tamura et al., 1977). Gabexate mesilate was shown to be effective in treating patients with DIC associated with sepsis (Taenaka et al., 1983). We previously demonstrated that gabexate mesilate reduced pulmonary vascular injury as well as coagulation abnormalities in rats administered endotoxin by inhibiting TNF-α production (Murakami et al., 1996). TNF-α plays a critical role in the development of acute respiratory distress syndrome (ARDS) by activating neutrophils in patients with sepsis.
(Shanley et al., 1995). Since ARDS is associated with a high mortality in patients with sepsis (St. John and Dorinsky, 1993), inhibition of TNF-α by gabexate mesilate could be useful in reducing the mortality of such patients by inhibiting both pulmonary vascular injury and coagulation abnormalities. However, the precise mechanism(s) by which gabexate mesilate inhibits TNF-α production by monocytes is not well understood at present.

Nuclear factor-κB (NF-κB), a transcription factor, is critically involved in the regulation of monocytic production of proinflammatory cytokines, such as TNF-α and interleukin-1β (Baldwin, 1996). The most abundant form of NF-κB is a heterodimer composed of p50 and p65 subunits (Baldwin, 1996). In unstimulated monocytes, NF-κB is localized in the cytosol as an inactive form bound to IκB (Finco and Baldwin, 1995). On stimulation with LPS, IκB undergoes phosphorylation, ubiquitination and proteolytic degradation, permitting NF-κB to translocate to the nucleus to initiate gene transcription (Baldwin, 1996).

Members of the mitogen-activated protein kinase (MAPK) family are also important in the signal transduction system for TNF-α transcription (Karin, 1995). Two subgroups of the MAPK family, c-Jun N-terminal kinase (JNK) and p38 MAPK, are involved in LPS-mediated expression of genes
encoding TNF-α (Hambleton et al., 1996; Lee et al., 1994). Activator protein-1 (AP-1), a transcription factor critically involved in the LPS-induced monocytic production of TNF-α is regulated by MAPK pathways (Karin, 1995). Moreover, stimulation of monocytes with LPS has been shown to enhance the transcriptional activity of AP-1 by activation of JNK and p38 MAPK (Whitmarsh and Davis, 1996).

Gabexate mesilate was shown to inhibit TNF-α production by inhibiting LPS-induced activation of NF-κB in human monocytes (Aosasa et al., 2001). However, little is known about the detailed molecular mechanisms by which gabexate mesilate inhibits the activation of NF-κB. Furthermore, it is not clear if gabexate mesilate inhibits the activation of AP-1 in human monocytes stimulated with LPS.

In the present study, we examined whether gabexate mesilate inhibits LPS-induced TNF-α production in human monocytes by inhibiting the activation of NF-κB and AP-1.
Materials and Methods

Materials

Gabexate mesilate was a generous gift from the Ono Pharmaceutical Company (Osaka, Japan). LPS (Escherichia coli, serotype 055:B5) was purchased from Difco (Detroit, MI). RPMI 1640 was obtained from Gibco BRL (Grand Island, NY). Supplemented calf serum (SCS) was from Hyclone (Logan, UT). Antibodies against IκBα, phosphorylated IκBα (Ser32), phosphorylated JNK (Thr183/Tyr185) and phosphorylated p38 MAPK (Thr180/Tyr182) were purchased from Cell Signaling Technology (Beverly, MA). DIG gel shift kit was from Roche Molecular Biochemicals (Mannheim, Germany). Double-stranded oligonucleotides with consensus sequences of NF-κB and AP-1 were obtained from Promega (Madison, WI). All other reagents used were of analytical grade.

Monocyte preparation and incubation

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy volunteer blood donors as described previously (Uchiba et al., 1997). The cell preparations were >90% monocytes, as determined by May-Giemsa staining. Cell viability was >95%, as determined by trypan blue dye exclusion test. Human monocytes were
incubated in RPMI 1640 supplemented with 1% SCS. The cells were stimulated with LPS in the presence or absence of gabexate mesilate.

Measurement of TNF-α

Human monocytes (5 x 10^5 cells/assay) were stimulated with LPS (100 ng/mL) or (1 ng/mL) for 4 h in the presence or absence of various concentrations of gabexate mesilate. Concentrations of TNF-α in supernatant fractions were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) kit for human TNF-α (Biosource International, Camarillo, CA).

Western blot analysis

Human monocytes (2 x 10^6 cells/assay) were stimulated with LPS (100 ng/mL) for indicated times in the presence or absence of gabexate mesilate (1.0 x 10^{-3} M). Cells were lysed in sodium dodecylsulfate (SDS) sample buffer (62.5 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mmol/L dithiothreitol, 0.1% bromphenol blue). Samples containing equal amounts of protein were resolved by 10% SDS polyacrylamide gel electrophoresis and then electroblotted onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, 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 ECL system (Amersham Pharmacia Biotech, Buckinghamshire, England).

Electrophoretic mobility shift assay (EMSA)

Human monocytes (1 x 10^7 cells/assay) were stimulated with LPS (100 ng/mL) for 1 h in the presence or absence of gabexate mesilate (1.0 x 10^-3 M). Nuclear extracts were prepared as described previously (Cheshire and Baldwin, 1997). Double-stranded oligonucleotides containing the sequences corresponding to NF-κB consensus site (5'-AGTTGAGGGGACTTTCCCAGGC-3' 3'-TCAACTCCCCTGAAAGGGTCCG-5') and AP-1 consensus site (5'-CGCTTGATGAGTCAGCCGGAA-3' 3'-GCGAACTACTCAGCCGAA-3') were 3'-end labeled with digoxigenin. Binding reactions were carried out in a final volume of 15 µL containing 0.8 ng of digoxigenin-labeled double-stranded NF-κB and AP-1 consensus oligonucleotides, 5 µg of nuclear extract, 1 µg of poly [d(I-C)] and binding buffer (20 mmol/L HEPES, pH 7.6, 1 mmol/L EDTA, 10 mmol/L (NH₄)₂SO₄, 1 mmol/L dithiothreitol, 2% Tween 20, 30 mmol/L KCl). The
mixtures were incubated for 15 min at room temperature, followed by another 10 min on ice. Samples were subjected to electrophoresis in 6% nondenaturing polyacrylamide gel in a 0.5 x Tris-borate-EDTA buffer system. The gel was transferred to a nylon membrane (Roche Molecular Biochemicals) by electroblotting. The membrane was then treated with antidigoxigenin-AP for 30 min and visualized using the chemiluminescent substrate CSPD (Roche Molecular Biochemicals).

Detection of specific binding of NF-κB and AP-1 to DNA by ELISA

Analysis of the specific binding of p65/p50 and c-Fos/c-Jun to their DNA consensus oligonucleotides was performed in nuclear extracts using the ELISA-based TransAM NF-κB p65/p50 and AP-1 c-Fos/c-Jun transcription factor assay kits (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. This method is based on non-isotopic quantitative ELISA-based analysis and was reported to be more sensitive than EMSA (Shen et al., 2002).

Statistical analysis

Data are presented as mean ± SD values. Results were compared by ANOVA followed by Scheffé's post hoc test. A level of $p < 0.05$ was
accepted as statistically significant.
Results

Effect of gabexate mesilate on TNF-α production by LPS-stimulated monocytes

Human monocytes were stimulated with LPS in the presence or absence of various concentrations of gabexate mesilate to examine the effect of gabexate mesilate on the TNF-α production. The LPS-induced increase in TNF-α production by monocytes was significantly inhibited by gabexate mesilate at a concentration of $1.0 \times 10^{-3}$ M (Fig. 1). Cell viability was not changed 4 h after incubation of monocytes with $1.0 \times 10^{-3}$ M of gabexate mesilate in the presence or absence of LPS (Data not shown).

Effect of gabexate mesilate on the LPS-induced increase of the binding of NF-κB to DNA

NF-κB is an important transcription factor in the induction of TNF-α transcription in response to LPS (Yao et al., 1997). We examined whether gabexate mesilate inhibited LPS-induced increase of the binding of NF-κB to DNA. Human monocytes were stimulated with LPS for 1 h in the presence or absence of gabexate mesilate. Nuclear extracts were assayed for NF-κB activation by EMSA using an oligonucleotide that contains the consensus NF-κB binding site. Analysis of the nuclear extract from LPS-
stimulated monocytes demonstrated an increase of the binding of NF-κB to DNA compared with the nuclear extract from unstimulated cells (Fig. 2). Pretreatment of monocytes with gabexate mesilate significantly inhibited LPS-induced increase of the binding of NF-κB to DNA (Fig. 2). We further analyzed the effect of gabexate mesilate on the LPS-induced increase of the specific binding of p65 and p50 to the consensus NF-κB site. As shown in Figs. 3A and 3B, the specific binding of p65 and p50 to DNA was significantly increased in monocytes stimulated with LPS compared with that seen in unstimulated cells. Pretreatment with gabexate mesilate significantly inhibited the LPS-induced increase of the specific binding of p65 and p50 to DNA (Figs. 3A and 3B).

Effect of gabexate mesilate on LPS-induced phosphorylation and degradation of IκBα

Activation of NF-κB was shown to require degradation of IκB, which normally binds to NF-κB in the cytoplasm to prevent nuclear translocation (Baldwin, 1996). To clarify whether gabexate mesilate inhibited the LPS-induced degradation of IκBα, we investigated the effect of gabexate mesilate on the cytoplasmic level of IκBα in monocytes stimulated with LPS by Western blot analysis. Treatment with LPS resulted
in the degradation of IκBα within 15 min, followed by an increase at 30 min after stimulation (Fig. 4A). Pretreatment of cells with gabexate mesilate inhibited IκBα degradation at 15 min following the addition of LPS (Fig. 4B).

To examine whether inhibition of LPS-induced IκBα degradation by gabexate mesilate was caused by suppression of IκBα phosphorylation, we determined the cytoplasmic level of the phosphorylated form of IκBα by Western blot analysis using an antibody against the phosphorylated form of IκBα. We were able to detect the phosphorylated form of IκBα prior to its degradation (Fig. 5A). Stimulation with LPS induced IκBα phosphorylation within 5 min, followed by a decrease of degradation of IκBα (Fig. 5A). Pretreatment with gabexate mesilate inhibited the phosphorylation of IκBα at 5 min after LPS stimulation (Fig. 5B). These results suggested that gabexate mesilate inhibited the binding of NF-κB to DNA by preventing IκBα phosphorylation and its subsequent degradation in LPS-stimulated human monocytes.

Effect of gabexate mesilate on LPS-induced increase of the binding of AP-1 to DNA
AP-1, a transcription factor, was shown to play an important role in the production of TNF-α in LPS-stimulated monocytes by increasing its transcription (Hambleton et al., 1996). Therefore, we also examined the effect of gabexate mesilate on LPS-induced binding of AP-1 to DNA. Human monocytes were treated with LPS for 1 h in the presence or absence of gabexate mesilate. Nuclear extracts were prepared and examined for AP-1 activation by EMSA using an oligonucleotide containing the consensus AP-1 binding site. As shown in Fig. 6, AP-1 binding to DNA was significantly increased in monocytes stimulated with LPS compared with that seen in unstimulated cells. Pretreatment with gabexate mesilate significantly inhibited LPS-induced binding of AP-1 to DNA (Fig. 6). We further analyzed the LPS-induced increase of the specific binding of c-Fos and c-Jun to the consensus AP-1 site of the target DNA. Specific binding of c-Fos and c-Jun to DNA was significantly increased in monocytes stimulated with LPS compared with that seen in unstimulated cells as shown in Figs. 7A and 7B, respectively. Pretreatment with gabexate mesilate significantly inhibited the LPS-induced increase of the specific binding of c-Fos and c-Jun to DNA (Figs. 7A and 7B).
p38 MAPK

Activation of JNK by LPS or proinflammatory cytokines was found to be prominently involved in the activation of AP-1 (Karin et al., 1997). To clarify whether gabexate mesilate inhibited LPS-induced AP-1 activation through inhibition of JNK phosphorylation, we investigated the effect of gabexate mesilate on phosphorylation of JNK at the cytoplasmic level in cells stimulated with LPS by Western blot analysis. Since dual phosphorylation of Thr183/Tyr185 of JNK is essential for the kinase activity (Derijard et al., 1994), we used an antibody that recognizes these phosphorylated residues in the analysis. Phosphorylation of JNK was increased after LPS stimulation, reaching its maximum level at 30 min after the stimulation, and then decreasing gradually (Fig. 8A). Pretreatment with gabexate mesilate inhibited JNK phosphorylation at 30 min after LPS stimulation (Fig. 8B).

Activation of p38 MAPK has been shown to contribute to AP-1 activation (Minden and Karin, 1997). Therefore, we examined the effect of gabexate mesilate on LPS-induced phosphorylation of p38 MAPK by Western blot analysis. Since dual phosphorylation at Thr180/Tyr182 is required for p38 MAPK activation (Raingeaud et al., 1995), we used an antibody that recognizes these phosphorylated residues in this analysis.
Stimulation of monocytes with LPS resulted in phosphorylation of p38 MAPK within 10 min. The cytoplasmic level of phosphorylated p38 MAPK reached a peak at 15 min after stimulation, and then gradually decreased (Fig. 9A). Gabexate mesilate significantly inhibited p38 MAPK phosphorylation at 15 min after LPS stimulation (Fig. 9B). These observations indicated that gabexate mesilate inhibited the binding of AP-1 to DNA by inhibiting phosphorylation of both JNK and p38 MAPK in LPS-stimulated human monocytes.

Effect of gabexate mesilate on TNF-α production and the increase in the binding of NF-κB to DNA in monocytes stimulated with a low concentration of LPS

We further examined whether various concentrations of gabexate mesilate lower than 1.0 x 10^{-3} M also inhibit LPS-induced TNF-α production in human monocytes stimulated with 1 ng/mL of LPS, a much lower concentration of LPS than that used in the present study. The LPS-induced increase in TNF-α production by monocytes was significantly inhibited by gabexate mesilate at the concentration of 1.0 x 10^{-6} M (Fig. 10), a concentration lower than that required to inhibit TNF-α production by monocytes stimulated with 100 ng/mL of LPS.
We also analyzed the effect of various concentrations of gabexate mesilate lower than $1.0 \times 10^{-3}$ M on the LPS-induced increase in the binding of NF-κB to DNA in human monocytes stimulated with 1 ng/mL of LPS. Nuclear extracts were analyzed for NF-κB activation by EMSA using an oligonucleotide that contains the consensus NF-κB binding site. As shown in Fig. 11, the binding of NF-κB to DNA was significantly increased in the nuclear extract from LPS-stimulated monocytes compared with that seen in the nuclear extract from unstimulated cells. Pretreatment of monocytes with various concentrations of gabexate mesilate lower than $1.0 \times 10^{-3}$ M significantly inhibited LPS-induced increase in the binding of NF-κB to DNA (Fig. 11).
Discussion

In the present study, we demonstrated that gabexate mesilate inhibited TNF-α production by LPS-stimulated human monocytes through inhibition of the activation of both NF-κB and AP-1.

Gabexate mesilate is a synthetic serine protease inhibitor which inhibits various serine proteases generated during the coagulation cascade and the inflammatory process (Tamura et al., 1977). Serine protease inhibitors, such as N-tosyl-L-phenylalanine chloromethyl ketone and N-benzoyl-L-tyrosine ethyl ester, prevented TNF-α production and TNF-α mRNA expression of macrophages induced by LPS by inhibiting NF-κB activity (Lo et al., 1997). Furthermore, Aosasa et al. (2001) showed that gabexate mesilate inhibited monocytic TNF-α production by inhibiting LPS-induced NF-κB activation. These findings are consistent with the observations in this study showing that gabexate mesilate inhibited LPS-induced binding of NF-κB to target sites of DNA in human monocytes.

The activity of NF-κB is primarily regulated by sequestration in the cytosol through anchoring to the inhibitory IκB proteins (Baldwin, 1996). Disruption of the NF-κB/IκB complex by phosphorylation, ubiquitination, and degradation of IκB allows subsequent translocation of NF-κB to the
nucleus (Baldwin, 1996). Our results demonstrated that gabexate mesilate prevented LPS-induced phosphorylation and subsequent degradation of IκBα. These observations strongly suggest that gabexate mesilate inhibited the LPS-induced activation of NF-κB by inhibiting degradation of IκB, thereby inhibiting TNF-α production in monocytes.

Since phosphorylated IκBα is degraded by a proteasome, a multi-subunit protease complex (Finco and Baldwin, 1995), it is possible that gabexate mesilate prevented the nuclear translocation of NF-κB by inhibiting degradation of IκB through inhibition of some proteases in the proteasome. In fact, proteasome inhibitors, such as N-benzyloxy carbonyl-Ile-Glu (O-t-Bu)-Ala-leucinal and N-acetyl-Leu-Leu-norleucinal were shown to inhibit LPS-induced degradation of IκBα and to block the production of TNF-α by human monocytes and by THP-1 cells (Haas et al., 1998).

The transcription factor AP-1 can also be activated by LPS, leading to enhancement of TNF-α transcription (Hambleton et al., 1996). We showed that gabexate mesilate inhibited LPS-induced binding of AP-1 to target sites of DNA in human monocytes. AP-1 has been identified as a target of MAPK signaling pathways (Karin, 1995). The responses to LPS or proinflammatory cytokines are mostly dependent on JNK and p38 MAPK
pathways, two MAPK cascades (Karin, 1995). Activation of JNK and p38 MAPK by dual phosphorylation was shown to enhance the transcriptional activity of AP-1 (Whitmarsh and Davis, 1996); while gabexate mesilate has been demonstrated to inhibit LPS-induced phosphorylation of JNK and p38 MAPK. The findings of this study suggest that gabexate mesilate inhibited AP-1 activation in LPS-stimulated human monocytes by preventing phosphorylation of both JNK and p38 MAPK.

Several lines of evidence from *in vitro* studies indicated that both toll-like receptor (TLR)-2 and TLR-4 expressed on the monocytic cell surface play critical roles in both activation of NF-κB and the expression of genes for various cytokines (Yang et al., 1998; Medzhitov et al., 1997). However, a previous study (Iwadou et al., 2002) demonstrated that gabexate mesilate inhibited TNF-α production in PBMCs stimulated with LPS without down-regulating TLR-4 expression, suggesting that the inhibitory effect of gabexate mesilate on LPS-induced TNF-α production could be due to the inhibition of intracellular signaling pathways. They also showed that gabexate mesilate did not down-regulate the expression of TLR-2 in PBMCs stimulated with Staphylococcal enterotoxin B (Iwadou et al., 2002), suggesting that expression of TLRs involved in the cytokine production by monocytes could not be affected by gabexate mesilate. Activation of NF-κB
and AP-1 may represent two distinct but interactive signal transduction pathways involved in LPS-induced inflammatory responses. Cross-talk occurs between the upstream pathways of NF-κB and MAPK (Stein et al., 1993). Thus, it is possible that gabexate mesilate inhibits LPS-induced phosphorylation of IκBα, JNK and p38 MAPK by inhibiting the upstream pathways. This possibility should be examined in future studies.

The chemical structure of gabexate mesilate is similar to that of CNI-1493, a tetravalent guanylhydrazone, that was shown to be a competitive inhibitor of cytokine-inducible L-arginine transport and nitric oxide production in macrophages activated with LPS and interferon-γ (Bianchi et al., 1995). CNI-1493 was also shown to inhibit the production of TNF-α by human monocytes (Bianchi et al., 1996) and this effect could be at least partly mediated by inhibition of p38 MAPK (Tracey, 1998). Since gabexate mesilate also inhibited activation of p38 MAPK in LPS-stimulated human monocytes as shown in the present study, these compounds might inhibit the monocytic TNF-α production by the similar molecular mechanism(s).

We demonstrated in rats that gabexate mesilate reduced ischemia/reperfusion-induced liver injury and compression trauma-induced spinal cord injury in which the ischemia/reperfusion mechanism is critically involved (Harada et al., 1999; Taoka et al., 1997). Reactive oxygen species
play an important role in the activation of monocytes to increase TNF-α production, thus contributing to the development of ischemia/reperfusion-induced tissue injury (Volk et al., 1999). AP-1 has been shown to be implicated in the ischemia/reperfusion-induced increase of TNF-α production (Yeh et al., 2000), and inhibition of AP-1 activation by gabexate mesilate might explain the therapeutic effect in the tissue injury induced by ischemia/reperfusion.

The concentration of gabexate mesilate required to inhibit LPS-induced TNF-α production in human monocytes was $1.0 \times 10^{-3}$ M when human monocytes were stimulated with LPS at a concentration of 100 ng/mL. The plasma level of gabexate mesilate in humans intravenously administered a therapeutic dose of gabexate mesilate (2 mg/kg/h) was $2.6 \times 10^{-7}$ M (Y. Sakai, unpublished observation), which is much lower than the concentration required to inhibit TNF-α production in vitro. Such a high concentration of gabexate mesilate ($1.0 \times 10^{-3}$ M) could not be attained in septic patients given a therapeutic dose of gabexate mesilate. In this study, human monocytes were stimulated with LPS at a concentration of 100 ng/mL which is much higher than that seen in plasma of septic patients (Opal et al., 1999). Gabexate mesilate inhibited both TNF-α production and binding of NF-κB to DNA at a concentration of $1.0 \times 10^{-6}$ M when
monocytes were stimulated with LPS at a concentration of 1 ng/mL. Thus, it is possible that gabexate mesilate inhibits the monocytic TNF-α production in septic patients whose plasma LPS levels might be much lower than 1 ng/mL.

In this study, human monocytes were incubated with gabexate mesilate 30 min before LPS stimulation. We previously demonstrated in rats that posttreatment as well as pretreatment of animals with gabexate mesilate prevented posttraumatic spinal cord injury in which TNF-α plays a causative role, suggesting that posttreatment of gabexate mesilate could be effective in vivo (Taoka et al., 1997). These observations strongly suggest that gabexate mesilate may be potential for inhibition of TNF-α production in the clinical setting.

Gabexate mesilate has been used to treat patients with DIC associated with sepsis probably due to its anticoagulant properties (Taenaka et al., 1983). Since TNF-α is critically involved in the activation of the extrinsic pathway of the coagulation system, thereby inducing DIC in the pathologic condition of sepsis (Okajima, 2001), inhibition of TNF-α production by gabexate mesilate might at least partly contribute to reduce the coagulation abnormalities in patients with sepsis. TNF-α also plays an important role in the development of pulmonary vascular injury by
activating neutrophils and endothelial cells (Zimmerman et al., 1999). Preliminary experiments showed that gabexate mesilate inhibited the activation of endothelial cells by inhibiting activation of NF-κB in cultured human umbilical vein endothelial cells. These observations indicated that inhibition of TNF-α production by gabexate mesilate might be useful in preventing the sepsis-associated organ failure such as ARDS, which adversely affects the outcome of patients with sepsis.

In conclusion, our results suggested that gabexate mesilate exerts its therapeutic effects in septic patients not only due to its anticoagulant activity, but also by inhibiting TNF-α production by monocytes. Such properties of gabexate mesilate might be useful in treating patients with sepsis for reducing organ failure as well as the coagulation abnormalities.
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Footnotes

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Figure Legends

Fig. 1. Effect of gabexate mesilate on TNF-α production by LPS-stimulated monocytes. Human monocytes were preincubated with various concentrations of gabexate mesilate for 30 min and then stimulated with LPS (100 ng/mL) for 4 h. Supernatants were collected and TNF-α levels were determined by ELISA. **, \( p < 0.01 \) versus LPS (-); ††, \( p < 0.01 \) versus LPS (+).

Fig. 2. Effect of gabexate mesilate on LPS-induced increase of the binding of NF-κB to DNA. Human monocytes were preincubated with or without gabexate mesilate (1.0 x 10^{-3} M) for 30 min and then stimulated with LPS (100 ng/mL) for 1 h. Cells were lysed and nuclei were isolated. Nuclear extracts (5 µg) were subjected to EMSA with a digoxigenin-labeled double-stranded oligonucleotide containing the consensus NF-κB binding site. Three independent experiments gave similar results and typical results were shown.

Fig. 3. Effect of gabexate mesilate on LPS-induced increase of the specific binding of p65 and p50 to DNA. Human monocytes were preincubated with
or without gabexate mesilate (1.0 × 10⁻³ M) for 30 min and then stimulated with LPS (100 ng/mL) for 1 h. Cells were lysed and nuclei were isolated. Specific binding of p65 and p50 to DNA was analyzed in nuclear extracts (5 µg) using ELISA as described in Materials and Methods. (A) Specific p65 binding to DNA. (B) Specific p50 binding to DNA. Three independent experiments gave similar results and typical results were shown. Data are expressed as relative activity of the specific binding normalized to that seen in unstimulated monocytes. **, p < 0.01 versus LPS (-); ††, p < 0.01 versus LPS (+).

Fig. 4. Effect of gabexate mesilate on LPS-induced degradation of IκBα. (A) Human monocytes were stimulated with LPS (100 ng/mL) for indicated times. Cytoplasmic extracts were prepared and examined by Western blot analysis using a polyclonal antibody against IκBα. Three independent experiments gave similar results and typical results were shown. (B) Human monocytes pretreated with or without gabexate mesilate (1.0 × 10⁻³ M) for 30 min were stimulated with LPS (100 ng/mL) for 15 min. Cytoplasmic extracts were prepared and examined by Western blot analysis using a polyclonal antibody against IκBα. Three independent experiments gave similar results and typical results were shown.
Fig. 5. Effect of gabexate mesilate on LPS-induced phosphorylation of IκBα. (A) Human monocytes were stimulated with LPS (100 ng/mL) for indicated times. Cytoplasmic extracts were analyzed by Western blot analysis using a phospho-specific IκBα antibody recognizing phosphorylation at Ser32. Three independent experiments gave similar results and typical results were shown. (B) Human monocytes pretreated with or without gabexate mesilate (1.0 x 10^{-3} M) for 30 min were stimulated with LPS (100 ng/mL) for 5 min. Cytoplasmic extracts were analyzed by Western blot analysis using a phospho-specific IκBα antibody recognizing phosphorylation at Ser32. Three independent experiments gave similar results and typical results were shown.

Fig. 6. Effect of gabexate mesilate on LPS-induced increase of the binding of AP-1 to DNA. Human monocytes were preincubated with or without gabexate mesilate (1.0 x 10^{-3} M) for 30 min and then stimulated with LPS (100 ng/mL) for 1 h. Cells were lysed and nuclei were isolated. Nuclear extracts (5 µg) were subjected to EMSA with a digoxigenin-labeled double-stranded oligonucleotide containing the consensus AP-1 binding site. Three independent experiments gave similar results and typical results were
shown.

Fig. 7. Effect of gabexate mesilate on LPS-induced increase of the specific binding of c-Fos and c-Jun to DNA. Human monocytes were preincubated with or without gabexate mesilate (1.0 x 10^{-3} M) for 30 min and then stimulated with LPS (100 ng/mL) for 1 h. Cells were lysed and nuclei were isolated. Specific binding of c-Fos and c-Jun to DNA was analyzed in nuclear extracts (5 µg) using ELISA as described in Materials and Methods. (A) Specific c-Fos binding to DNA. (B) Specific c-Jun binding to DNA. Three independent experiments gave similar results and typical results were shown. Data are expressed as relative activity of the specific binding normalized to that seen in unstimulated monocytes. **, p < 0.01 versus LPS (-); ††, p < 0.01 versus LPS (+).

Fig. 8. Effect of gabexate mesilate on LPS-induced phosphorylation of JNK. (A) Human monocytes were stimulated with LPS (100 ng/mL) for indicated times. The cytoplasmic amounts of phosphorylated JNK were determined by Western blot analysis using a phospho-specific JNK antibody recognizing phosphorylation at Thr183/Tyr185. Three independent experiments gave similar results and typical results were shown. (B) Human monocytes
pretreated with or without gabexate mesilate (1.0 x 10^{-3} M) for 30 min were stimulated with LPS (100 ng/mL) for 30 min. The cytoplasmic amounts of phosphorylated JNK were determined by Western blot analysis using a phospho-specific JNK antibody recognizing phosphorylation at Thr183/Tyr185. Three independent experiments gave similar results and typical results were shown.

Fig. 9. Effect of gabexate mesilate on LPS-induced phosphorylation of p38 MAPK. (A) Human monocytes were stimulated with LPS (100 ng/mL) for indicated times. The cytoplasmic levels of phosphorylated p38 MAPK were determined by Western blot analysis using a phospho-specific p38 MAPK antibody recognizing phosphorylation at Thr180/Tyr182. Three independent experiments gave similar results and typical results were shown. (B) Human monocytes were preincubated with or without gabexate mesilate (1.0 x 10^{-3} M) for 30 min and then stimulated with LPS (100 ng/mL) for 15 min. The cytoplasmic levels of phosphorylated p38 MAPK were determined by Western blot analysis using a phospho-specific p38 MAPK antibody recognizing phosphorylation at Thr180/Tyr182. Three independent experiments gave similar results and typical results were shown.
Fig. 10. Effect of gabexate mesilate on TNF-α production by monocytes stimulated with a low concentration of LPS. Human monocytes were preincubated with various concentrations of gabexate mesilate for 30 min and then stimulated with LPS (1 ng/mL) for 4 h. Supernatants were collected and TNF-α levels were determined by ELISA. **, $p < 0.01$ versus LPS (-); ††, $p < 0.01$ versus LPS (+).

Fig. 11. Effect of gabexate mesilate on increase of the binding of NF-κB to DNA in monocytes stimulated with a low concentration of LPS. Human monocytes were preincubated with various concentrations of gabexate mesilate for 30 min and then stimulated with LPS (1 ng/mL) for 1 h. Cells were lysed and nuclei were isolated. Nuclear extracts (5 µg) were subjected to EMSA with a digoxigenin-labeled double-stranded oligonucleotide containing the consensus NF-κB binding site. Three independent experiments gave similar results and typical results were shown.
**Fig. 2**

NF-κB →

<table>
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<tr>
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<th>+</th>
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<tr>
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<td>-</td>
<td>-</td>
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<tr>
<td>mesilate</td>
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[Image of gel with bands and labels]
Fig. 3

(A) DNA binding activity of p65 (Relative activity)

(B) DNA binding activity of p50 (Relative activity)

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<tr>
<td>LPS (+) (n=3)</td>
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<tr>
<td>LPS + Gabexate mesilate (n=3)</td>
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** Differences indicate significant differences from the control. †† Differences indicate significant differences from the LPS (+) condition.
Fig. 4

A

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B

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**Fig. 5**

**A**

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</tbody>
</table>

**B**

| phospho-IκBα → |   |   |    | 5 min |
| LPS            | - | + | +  |       |
| Gabexate mesilrate | - | - | +  |       |
Fig. 6

AP-1 →

LPS  Gabexate mesilate

- + +
- - +
Fig. 8

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|                   |   |   |    |    |    |    |
|                   | phospho-p54 | → | phospho-p46 | → |

B

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|                   |   |   |    |    |    |    |
|                   | phospho-p54 | → | phospho-p46 | → |

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30 min
Fig. 9

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B

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Fig. 10

![Graph showing TNF-α (ng/1.0 x 10^6 Cells) levels with LPS and Gabexate mesilate (M) concentrations.](image)
Fig. 11

NF-κB →

<table>
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<tr>
<th>LPS</th>
<th>Gabexate mesilate (M)</th>
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