

Gabexate mesilate, a synthetic anticoagulant, inhibits the expression of endothelial leukocyte adhesion molecules *in vitro**

Mitsuhiro Uchiba, MD; Kenji Okajima, MD; Christoph Kaun, BSc; Bernd R. Binder, MD; Johann Wojta, PhD

Objective: Gabexate mesilate, a synthetic protease inhibitor, has been shown to reduce endotoxin-induced pulmonary vascular injury in an animal model of sepsis by inhibiting leukocyte activation. We examined whether gabexate mesilate inhibits tumor necrosis factor- α -induced expression of leukocyte adhesion molecules in cultured endothelial cells.

Design: Prospective, randomized, controlled study.

Setting: Research laboratory at a university medical center

Subjects: Cultured human umbilical vein endothelial cell (HUVECs).

Interventions: HUVECs were stimulated with tumor necrosis factor- α or lipopolysaccharide in the presence or absence of gabexate mesilate. Expression of E-selectin and intercellular adhesion molecule-1 was measured by cellular enzyme-linked immunosorbent assay. Messenger RNA levels of E-selectin and intercellular adhesion molecule-1 were determined by reverse transcription-polymerase chain reaction. DNA-binding activity of p65 in the nuclear extracts was evaluated by enzyme-linked immunosorbent assay. Nuclear translocation of nuclear factor- κ B induced by tumor necrosis factor- α was evaluated by immunocytochemistry and Western blot analysis. Degradation and phosphorylation of inhibitor of nuclear factor- κ B (I κ B) induced by tumor necrosis factor- α were evaluated by Western blot analysis.

Measurements and Main Results: Gabexate mesilate inhibited the tumor necrosis factor- α -induced increases in the endothelial expression of E-selectin and intercellular adhesion molecule-1 by inhibiting the transcription. Tumor necrosis factor- α -induced increase in DNA binding of p65 was inhibited by gabexate mesilate through inhibition of the nuclear translocation of p65. Gabexate mesilate inhibited the tumor necrosis factor- α -induced degradation of I κ B α , an inhibitor of nuclear factor- κ B, by inhibiting phosphorylation of I κ B α in HUVECs.

Conclusions: Gabexate mesilate inhibited the expression of leukocyte adhesion molecules by inhibiting the nuclear factor- κ B-mediated transcription in HUVECs. Inhibition of nuclear factor- κ B activation by gabexate mesilate could be explained by inhibition of degradation of I κ B. Gabexate mesilate might reduce lipopolysaccharide-induced pulmonary vascular injury not only by inhibiting monocytic tumor necrosis factor- α production but by inhibiting the expression of endothelial leukocyte adhesion molecules. (Crit Care Med 2003; 31:1147-1153)

KEY WORDS: gabexate mesilate; disseminated intravascular coagulation; acute respiratory distress syndrome; E-selectin; intercellular adhesion molecule-1; nuclear factor- κ B

Lipopolysaccharide (LPS) and tumor necrosis factor- α (TNF- α) play important roles in the development of acute respiratory distress syndrome (ARDS) and disseminated intravascular coagulation (DIC) in patients with sepsis (1). TNF- α is released from monocytes stimulated by LPS and activates the coagulation system, leading to microthrombus formation (2). TNF- α also plays a critical role in

the development of LPS-induced pulmonary vascular injury by activating both neutrophils and endothelial cells (3). Activated neutrophils release a wide variety of inflammatory mediators such as neutrophil elastase and oxygen free radicals that are capable of damaging endothelial cells, thereby contributing to the development of pulmonary vascular injury (4, 5). Both LPS and TNF- α increase the expression of endothelial leukocyte adhesion molecules, such as E-selectin and intercellular adhesion molecule-1 (ICAM-1).

Nuclear factor- κ B (NF- κ B), a transcription factor, is critically involved in the processes leading to the TNF- α -induced expression of E-selectin and ICAM-1 in endothelial cells (6, 7). The most abundant form of NF- κ B is a heterodimer composed of p50 and p65 subunits (8). In unstimulated endothelial cells, NF- κ B is localized in the cytosol as an inactive form bound to inhibitor of NF- κ B (I κ B) (8).

TNF- α , released by activated monocytes, stimulates endothelial cells by binding to TNF- α receptor-1 (TNFR-1) on the cell surface, leading to the activation of I κ B kinase, an enzyme responsible for phosphorylation of I κ B (9). Consequently, I κ B undergoes phosphorylation, ubiquitination, and proteolytic degradation, permitting NF- κ B to translocate to the nucleus to initiate the transcription of E-selectin and ICAM-1 gene (6, 7, 9).

These leukocyte adhesion molecules enable activated neutrophils to adhere to endothelial cells (10). Recruitment of neutrophils through up-regulation of these endothelial adhesion molecules has been considered to be a critical mechanism of ARDS in sepsis (11).

Gabexate mesilate (GM) is a synthetic serine protease inhibitor that inhibits various serine proteases generated during the coagulation cascade and the inflammatory process (12). GM

*See also p. 1284.

From the Department of Laboratory Medicine (MU, KO), Kumamoto University School of Medicine, Kumamoto, Japan; and the Institute of Vascular Biology and Thrombosis Research (CK, BRB, JW), University of Vienna, Vienna, Austria.

Address requests for reprints to: Kenji Okajima, MD, Department of Laboratory Medicine, Kumamoto University School of Medicine, Honjo 1-1-1, Kumamoto, 860-0811, Japan. E-mail: whynot@kaiju.medic.kumamoto-u.ac.jp

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has been used to treat patients with DIC due to its anticoagulant properties, and it was shown to be effective in treating patients with DIC associated with sepsis (13). We previously reported that GM reduces LPS-induced coagulation abnormalities and pulmonary vascular injury by inhibiting TNF- α production in rats (14), suggesting that GM might reduce both DIC and ARDS in patients with sepsis. Since ARDS is a critical pathologic condition that adversely affects the outcome of septic patients associated with DIC (15), attenuation of activated neutrophil-induced pulmonary vascular injury by some therapeutic agents including GM would be useful to reduce the mortality rate of such patients.

Although increases in the expression of endothelial leukocyte adhesion molecules induced by LPS and TNF- α could be implicated in the development of sepsis-induced pulmonary vascular injury (11), whether GM inhibits the increase in the expression of endothelial leukocyte adhesion molecules is not known. In the present study, we investigated the effect of GM on the increase in the expression of E-selectin and ICAM-1 by using cultured human umbilical vein endothelial cells (HUVECs). In addition, we further investigated the mechanisms by which GM regulates the endothelial expression of leukocyte adhesion molecules in HUVECs.

METHODS

Cell Culture. Endothelial cells were isolated from fresh human umbilical cord veins with collagenase by a technique similar to that described by Jaffe et al. (16). Cells from four to six cords were pooled and grown to confluence at 37°C in humidified 95% air-5% CO₂ atmosphere in M199 supplemented with 10% heat-inactivated supplemented calf serum (SCS, HyClone, Logan, UT), 100 μ g/mL streptomycin, 100 IU/mL penicillin, 250 ng/mL amphotericin B, 1 mM glutamine, 5 IU/mL heparin, and 50 μ g/mL endothelial cell growth supplement.

Cellular Enzyme-Linked Immunosorbent Assay (ELISA) for E-Selectin and ICAM-1. Surface expression of adhesion molecules (E-selectin and ICAM-1) on HUVECs was quantified by ELISAs as described previously (17). In brief, HUVECs were seeded into a 96-well plate to reach confluency the next day. Cells were incubated for 30 mins with M199 containing 1% SCS with or without GM (Ono Pharmaceutical, Osaka, Japan). The cells were stimulated by human recombinant TNF- α (10 units/mL, Boehringer Mannheim, Mannheim,

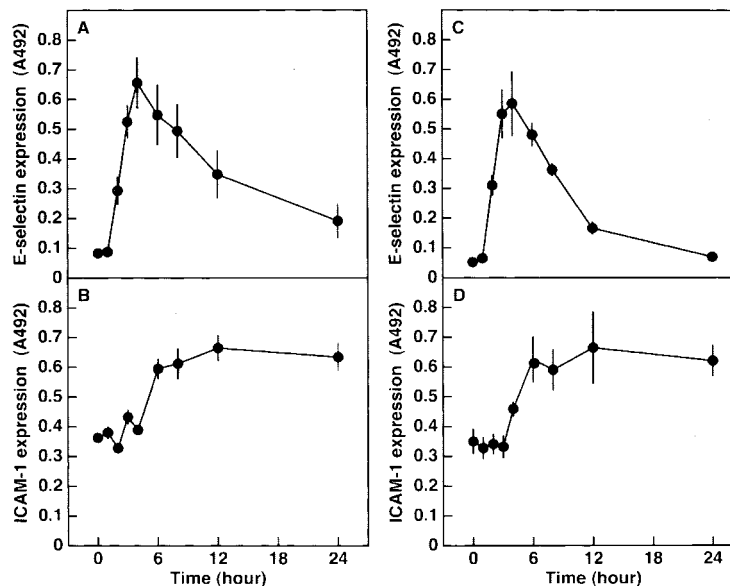


Figure 1. Effects of lipopolysaccharide (LPS) and tumor necrosis factor (TNF)- α on the expression of E-selectin and intercellular adhesion molecule (ICAM)-1 in human umbilical vein endothelial cells (HUVECs). HUVECs were cultured in M199/1% supplemented calf serum (SCS) with LPS (10 μ g/mL). At indicated time points after stimulation with LPS, the endothelial expression of E-selectin (A) and ICAM-1 (B) was measured by using cellular enzyme-linked immunosorbent assay (ELISA). HUVECs were cultured in M199/1% SCS with TNF- α (10 units/mL). Expression of E-selectin (C) and ICAM-1 (D) on HUVECs was measured by using cellular ELISA method at indicated time points after stimulation with TNF- α . Values are expressed as the mean \pm SD of triplicate in one experiment representative of three performed with similar results.

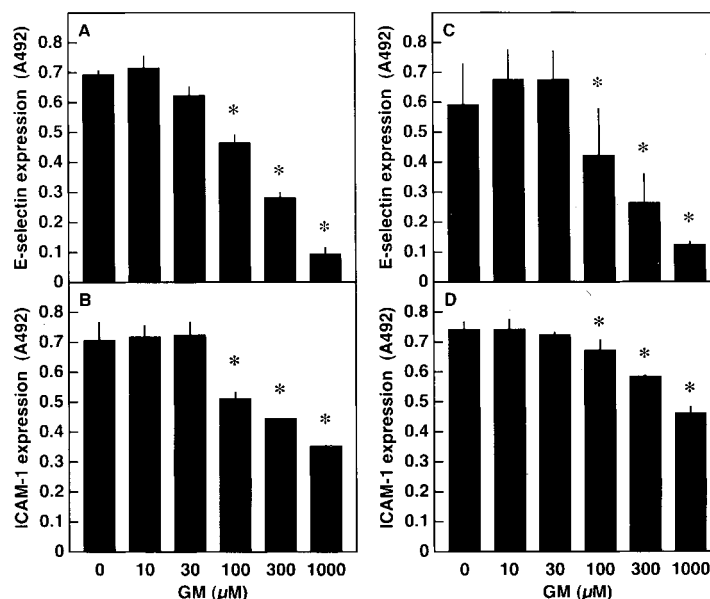


Figure 2. Effect of gabexate mesilate (GM) on the lipopolysaccharide (LPS)- and tumor necrosis factor (TNF)- α -induced expression of E-selectin and intercellular adhesion molecule (ICAM)-1 in human umbilical vein endothelial cells (HUVECs). Endothelial expression of E-selectin and ICAM-1 was determined by cellular enzyme-linked immunosorbent assay by using anti-E-selectin and anti-ICAM-1 antibodies. HUVECs were stimulated with LPS (10 μ g/mL) (A, B) or TNF- α (10 units/mL) (C, D). GM was added to culture media 30 mins before stimulation. E-selectin and ICAM-1 were measured 4 hrs and 6 hrs after stimulation, respectively. Values are expressed as the mean \pm SD of triplicate in one experiment representative of three performed with similar results. * p < .01 vs. LPS or TNF- α without GM.

Germany) or LPS (*Escherichia coli*, serotype 055:B5, 10 $\mu\text{g}/\text{mL}$, Difco, Detroit, MI). Adhesion molecules were detected by using cellular ELISAs by incubating cells with a primary antibody to either E-selectin or ICAM-1 (R&D Systems, Minneapolis, MN) and a horseradish peroxidase-conjugated secondary antibody. The expression of E-selectin and ICAM-1 was quantitated by the addition of the peroxidase substrate o-phenyldiamine. The absorbance of each well was measured at 492 nm in an automated reader.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction. Two hours after TNF- α stimulation, RNA was extracted from HUVECs by using a chloroform phenol extraction kit (RNAzol B, Tel-Test, Friendswood, TX). RNA quantity and quality were measured spectrophotometrically at 260/280 nm. An extinction coefficient of 12 $\text{mg}\cdot\mu\text{L}^{-1}\cdot\text{OD}^{-1}\cdot\text{cm}^{-1}$ at 260 nm was used to calculate RNA concentrations.

All reagents for complementary DNA synthesis and amplification were obtained from Life Technologies (Gaithersburg, MD). One microgram of total RNA was used for complementary DNA synthesis by using SuperScript II RT and a poly dTTP primer. Polymerase chain reactions were conducted in Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA). For polymerase chain reaction, the following primers were used: E-selectin, 5'-AGAAATATGTGGTTCCACGATGA-3' and 5'-AAACTGGAGATTCCTTTGGAATTG-3'; ICAM-1, 5'-AACCGAAGGTGTATGAACTG-3' and 5'-CGAGGTGTTCTCAAACAGCTC-3'; and GAPDH, 5'-ATGACAACAGCCTCAAGATCATCAG-3' and 5'-CTGGTGGTCCAGGGGTCTTACTCCT-3' (18). Complementary DNA products were separated on 2% agarose gels in Tris-acetate-EDTA buffer containing 40 mM Tris-acetate, 2 mM Na_2EDTA , pH 8.5, in the presence of 1 mg/mL ethidium bromide. Densitometric quantification of the bands followed with NIH Image 1.62 software.

Preparation of Whole Cell Lysate and Nuclear Extract. Whole cell lysates were prepared by using lysate buffer (50 mM Tris, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, 1 mM sodium vanadate, 50 mM sodium fluoride, 2 mM EDTA [pH 8.0], 1 mM phenylmethylsulfonyl fluoride, 10 mg/mL leupeptin, 10 mg/mL pepstatin A, 10 mg/mL aprotinin). Nuclear extracts from confluent HUVECs were prepared as described previously (17). Protein concentrations in the extract were determined by using a protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Sodium dodecyl sulfate, glycerol, and bromophenol blue were added to the whole cell lysates and nuclear extracts (17). Whole cell lysates and nuclear extracts were denatured by heating (60°C, 20 mins) and sonicated 30 secs before sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Measurement of DNA-Binding Activity of p65. One hour after TNF- α stimulation, nuclear extract of HUVECs were collected as de-

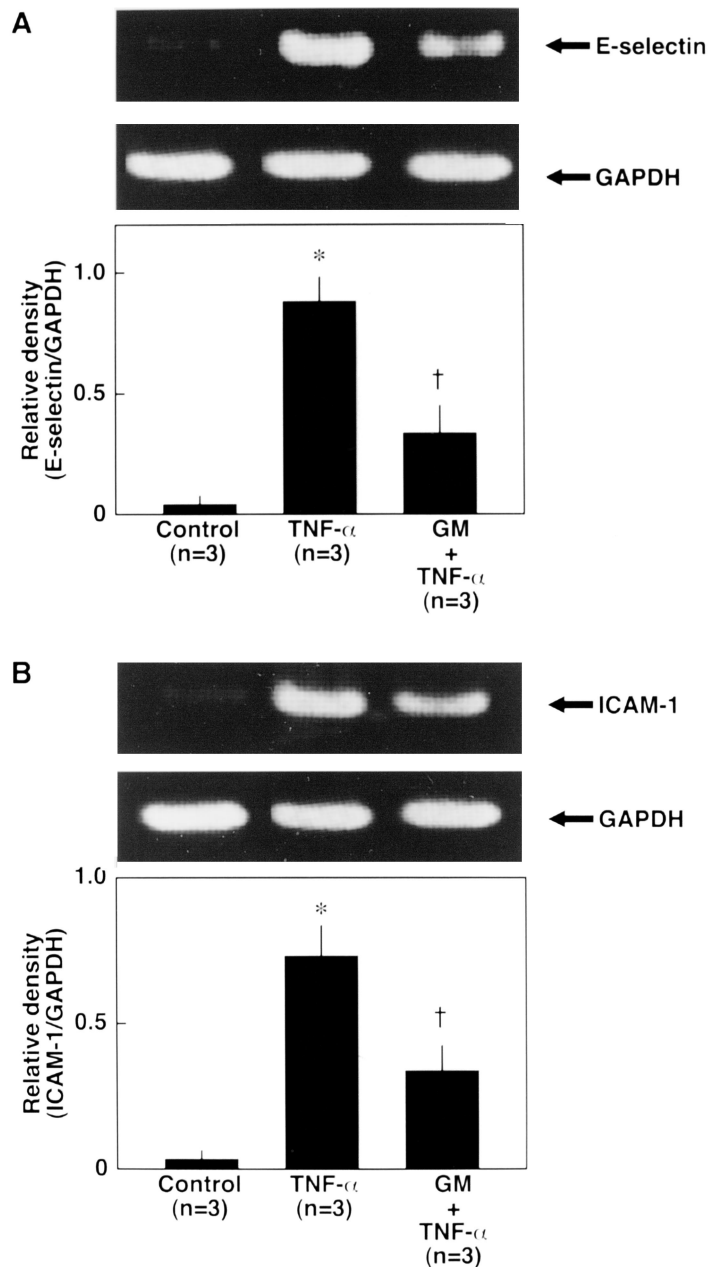


Figure 3. Effect of gabexate mesilate (GM) on tumor necrosis factor (TNF)- α -induced increase in E-selectin and intercellular adhesion molecule (ICAM)-1 messenger RNA (mRNA) levels in human umbilical vein endothelial cells (HUVECs). HUVECs were stimulated with TNF- α (10 units/mL) for 2 hrs. Total mRNA of HUVECs was collected as described in Methods. GM (1.0×10^{-4} M) was added 30 mins before TNF- α stimulation. mRNA levels of E-selectin, ICAM-1, and glyceraldehyde phosphate dehydrogenase (GAPDH) were evaluated by reverse transcription-polymerase chain reaction as described in Methods. Values are expressed as the mean \pm SD of triplicate in one experiment representative of three performed with similar results. * $p < .01$ vs. control, † $p < .01$ vs. TNF- α without GM.

scribed previously. DNA-binding activity of p65 in nuclear extract was evaluated by ELISA-based method (Mercury TransFactor, Clontech Laboratories, Palo Alto, CA).

Western Blotting. HUVECs were treated with either vehicle or GM for 30 mins at 37°C before the addition of TNF- α (10 units/mL). Various times after TNF- α stimulation, whole cell extracts and nuclear extracts were pre-

pared as described previously. Ten micrograms of protein was charged in each well, separated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a PVDF membrane. Total I κ B α protein, phosphorylated I κ B α protein, and p65 were detected according to the ECL protocol by using anti-I κ B α immunoglobulin (Ig) G (Cell Signaling Technology, Beverly, MA),

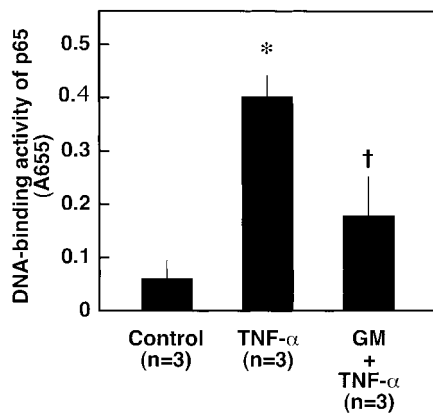


Figure 4. Effect of gabexate mesilate (GM) on tumor necrosis factor (TNF)- α -induced increase in DNA-binding activity of p65. Human umbilical vein endothelial cells (HUVECs) were stimulated with TNF- α (10 units/mL) for 1 hr. Nuclear extract of HUVECs were collected as described in Methods. GM (1.0×10^{-4} M) was added 30 mins before TNF- α stimulation. DNA-binding activity of p65 was evaluated by enzyme-linked immunosorbent assay base method. Values are expressed as the mean \pm SD of triplicate in one experiment representative of three performed with similar results. * $p < .01$ vs. control, † $p < .01$ vs. TNF- α without GM.

phosphorylated-I κ B α IgG (Cell Signaling Technology), and anti-p65 IgG (Santa Cruz, Santa Cruz, CA), respectively. Densitometric quantification of the bands followed with NIH Image 1.62 software.

Immunofluorescence Assay. Immunofluorescence assay was performed as previously described (17). Briefly, cells were grown in LabTek tissue-culture chamber slides (Nunc, Naperville, IL) for ≥ 24 hrs before fixation. One hour after TNF- α stimulation, cells were fixed and incubated with anti-p65 IgG. After 1 hr of incubation, cells were washed in phosphate-buffered saline and incubated with fluorescein isothiocyanate-labeled goat anti-rabbit IgG.

Cell Viability. HUVECs were cultured in 24-well plates. After confluency, HUVECs were stimulated LPS or TNF- α with or without GM. After 4 hrs and 6 hrs stimulation of LPS or TNF- α , HUVECs were trypsinized and the cell number counted. Cell viability was evaluated by using a trypan blue dye exclusion method (19).

Statistical Analysis. Data are presented as mean \pm SD. Results were compared by using analysis of variance and the Scheffe's *post hoc* test. A level of $p < .05$ was accepted as statistically significant.

RESULTS

Effect of GM on the Increases in the Expression of E-Selectin and ICAM-1 in HUVECs Stimulated With LPS and

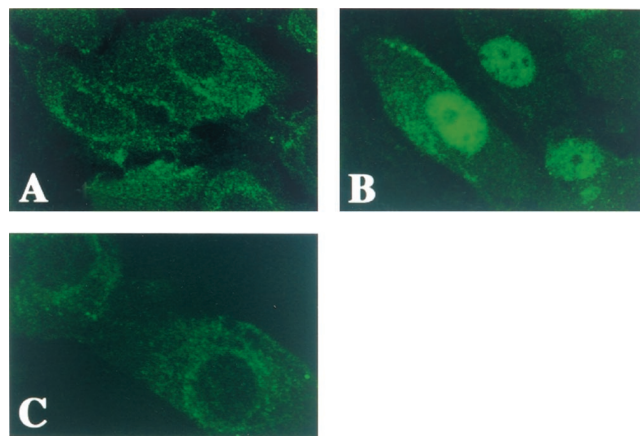


Figure 5. Immunofluorescence staining of p65 in unstimulated human umbilical vein endothelial cells (HUVECs; A), tumor necrosis factor (TNF)- α -treated HUVECs (B), and gabexate mesilate (GM) plus TNF- α -treated HUVECs (C). HUVECs were stimulated with TNF- α (10 units/mL) for 1 hr before cell fixation. GM (1.0×10^{-4} M) was added 30 mins before TNF- α stimulation. Immunofluorescence staining was performed as described in Methods.

TNF- α . Expression of E-selectin in HUVECs began to increase 30 mins after stimulation with LPS, reaching its maximum at 4 hrs after stimulation, followed by a gradual decrease (Fig. 1A). The expression of ICAM-1 began to increase 4 hrs after stimulation with LPS, reaching its maximum at 6 hrs after stimulation, and remained high until 24 hrs after stimulation (Fig. 1B). Similar changes in the expression of these adhesion molecules were observed after stimulation with TNF- α (Fig. 1C and 1D). When preincubated with HUVECs, GM at concentrations higher than 1.0×10^{-4} M significantly inhibited the increases in the expression of E-selectin and ICAM-1 at 4 and 6 hrs after stimulation with LPS, respectively (Fig. 2A and 2B). Similar inhibition by GM was found in the expression of these adhesion molecules in HUVECs stimulated with TNF- α (Fig. 2C and 2D). The number of living cells was not decreased in the presence of GM (1×10^{-3} M) 4 hrs and 6 hrs after stimulation with LPS and TNF- α , respectively (data not shown).

Effect of GM on TNF- α -Induced Increases in Messenger RNA Levels of E-Selectin and ICAM-1 in HUVECs. Messenger RNA levels of E-selectin and ICAM-1 were significantly increased in HUVECs 2 hrs after stimulation with TNF- α (Fig. 3). Pretreatment of HUVECs with GM (1.0×10^{-4} M) significantly inhibited the TNF- α -induced increases in messenger RNA levels of E-selectin and ICAM-1 (Fig. 3).

Effect of GM on TNF- α -Induced Activation Process of NF- κ B. Since the TNF- α -induced increases in the expression of E-selectin and ICAM-1 are mediated by activation of NF- κ B, a transcription factor (6, 7), we investigated the effect of GM on the NF- κ B activation in HUVECs stimulated with TNF- α . DNA-binding activity of p65, a subunit of NF- κ B, in nuclear extract was significantly increased in HUVECs 1 hr after stimulation with TNF- α (Fig. 4).

We examined the effect of GM on the TNF- α -induced nuclear translocation of p65 in HUVECs by using immunofluorescence staining with anti-p65 antibody 1 hr after stimulation with TNF- α . In unstimulated HUVECs, p65 was localized almost exclusively in cytoplasm of HUVECs (Fig. 5A). Stimulation of HUVECs with TNF- α resulted in the nuclear translocation of p65 1 hr after the stimulation (Fig. 5B). However, pretreatment of HUVECs with GM (1.0×10^{-4} M) significantly inhibited the TNF- α -induced nuclear translocation of p65 (Fig. 5C).

When examined with Western blot analysis, TNF- α -induced increase in the nuclear levels of p65 was inhibited by pretreatment of HUVECs with GM (1.0×10^{-4} M) (Fig. 6).

Since translocation of NF- κ B from the cytosol to the nucleus is induced by the degradation of I κ B, an inhibitor of NF- κ B (8), we examined the effect of GM on the degradation of I κ B α , a subunit of I κ B, in HUVECs stimulated with TNF- α by using Western blot analysis. The intracellular level of I κ B α began to

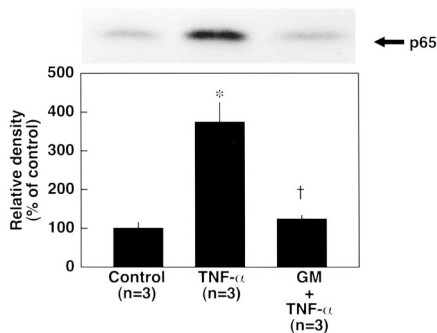


Figure 6. Effect of gabexate mesilate (GM) on tumor necrosis factor (TNF)- α -induced translocation of p65 in human umbilical vein endothelial cells (HUVECs). HUVECs pretreated with and without GM (1.0×10^{-4} M) for 30 mins were stimulated with TNF- α for 1 hr (10 units/mL). Nuclear extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and determined for p65 level by Western blot analysis as shown in the upper panel. The lower panel shows the result of nuclear levels of p65 by densitometry analysis. Results were normalized by mean densitometric level of control (without TNF- α stimulation) to 100%. Shown is the mean \pm SD (n = 3). * $p < .01$ vs. control, † $p < .01$ vs. TNF- α . The result is representative of three experiments with similar results.

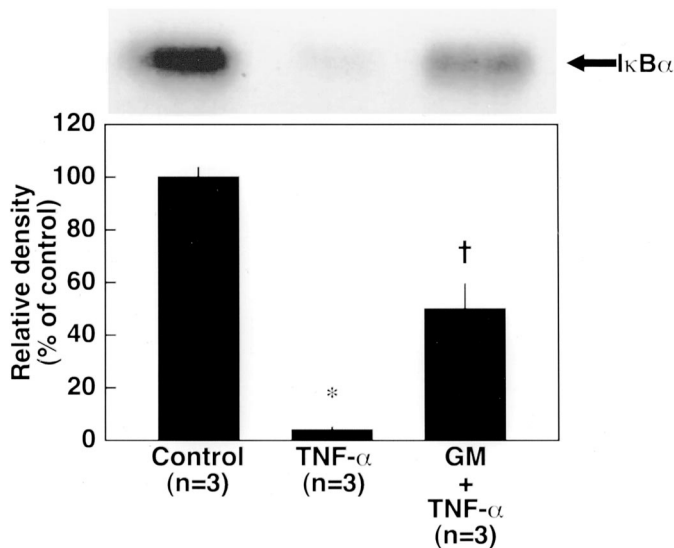


Figure 8. Effect of gabexate mesilate (GM) on tumor necrosis factor (TNF)- α -induced degradation of inhibitor of nuclear factor- κ B (I κ B α). Human umbilical vein endothelial cells pretreated for 30 mins with or without GM (1.0×10^{-4} M) were incubated for 30 mins with TNF- α (10 units/mL). Whole cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and determined for I κ B α levels by Western blot analysis as shown in the upper panel. The lower panel shows the result of intracellular levels of I κ B α by densitometry analysis. Results were normalized by mean densitometric level of control (without TNF- α stimulation) to 100%. Shown is the mean \pm SD (n = 3). * $p < .01$ vs. control, † $p < .01$ vs. TNF- α . The result is representative of three experiments with similar results.

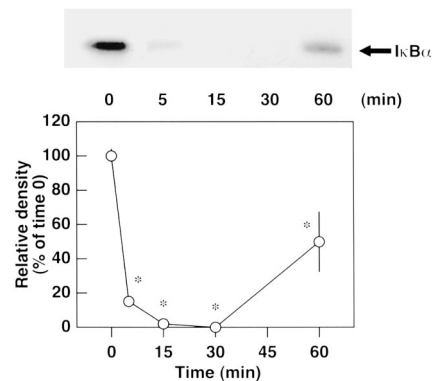


Figure 7. Effect of tumor necrosis factor (TNF)- α on inhibitor of nuclear factor- κ B (I κ B α) degradation in human umbilical vein endothelial cells (HUVECs). HUVECs were treated with TNF- α (10 units/mL). At indicated time points, whole cell lysates were collected and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis as described in Methods. I κ B α levels were determined by Western blot analysis as shown in the upper panel. The lower panel shows the result of intracellular levels of I κ B α by densitometry analysis. Results were normalized by mean densitometric level of time 0 to 100%. Shown is the mean \pm SD (n = 3). * $p < .01$ vs. time 0. The result is representative of three experiments with similar results.

decrease 5 mins after stimulation of HUVECs with TNF- α , reaching its minimum level at 30 mins after the stimu-

lation, and then increased gradually (Fig. 7). GM (1.0×10^{-4} M) inhibited the decrease in the cellular level of I κ B α 30 mins after the stimulation of HUVECs with TNF- α (Fig. 8).

Effect of GM on TNF- α -Induced Phosphorylation of I κ B α . Degradation of I κ B was initiated by phosphorylation of I κ B by I κ B kinase (8). As shown in Figure 9, the intracellular level of phosphorylated I κ B α , as detected by Western blot analysis, began to increase 5 mins after stimulation with TNF- α , reaching its maximum level at 15 mins after the stimulation, and decreased thereafter. Pretreatment with GM (1.0×10^{-4} M) significantly inhibited the increase in the intracellular level of phosphorylated I κ B α 15 mins after the stimulation of HUVECs with TNF- α (Fig. 10).

DISCUSSION

In the present study, we demonstrated that GM inhibited the expression of E-selectin and ICAM-1 in HUVECs stimulated with LPS and TNF- α . The E-selectin and ICAM-1 genes contain sequences in their promoter regions that are recognized by nuclear factors (6, 20). Several lines of evidence have demonstrated that the NF- κ B sites are functional elements in

TNF- α - and LPS-induced expression of E-selectin and ICAM-1 in endothelial cells (21, 22). Thus, GM might inhibit the LPS- and TNF- α -induced expression of these two adhesion molecules by inhibiting activation of NF- κ B, a heterodimer consisting of p65 and p50, in HUVECs (6, 7). Consistent with this hypothesis, TNF- α -induced nuclear translocation of p65 was significantly inhibited by GM in HUVECs as shown by immunocytochemical and Western blot analysis.

Thus, GM might inhibit the nuclear translocation of NF- κ B by inhibiting the degradation of I κ B. The present observation that GM inhibited the TNF- α -induced degradation of I κ B α , a subunit of I κ B, in HUVECs is consistent with this hypothesis. In addition, we further examined how GM inhibited the degradation of I κ B α in HUVECs stimulated with TNF- α . Since phosphorylated I κ B α is degraded by proteasome complex (8), a serine protease inhibitor, such as GM, might inhibit the degradation of I κ B by inhibiting some serine proteases in the proteasome complex. In fact, proteasome inhibitors such as lactacystin and PS-341 have been shown to inhibit TNF- α -induced expression of E-selectin and ICAM-1 in

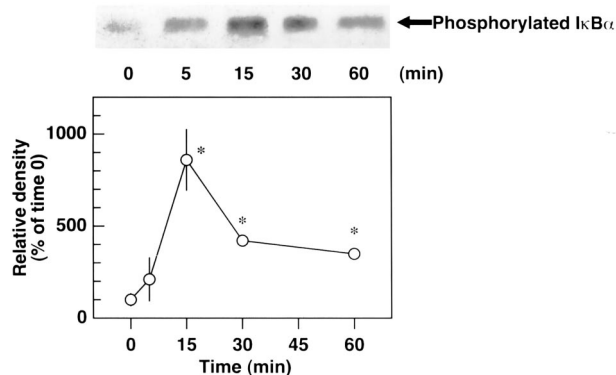


Figure 9. Effect of tumor necrosis factor (TNF)- α on the phosphorylation of inhibitor of nuclear factor- κ B ($I\kappa B\alpha$) in human umbilical vein endothelial cells (HUVECs). HUVECs were treated with TNF- α (10 units/mL). At the indicated time points, whole cell lysates were collected and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis as described in Methods. $I\kappa B\alpha$ phosphorylation was determined by Western blot analysis using a specific antibody against phosphorylated- $I\kappa B\alpha$ as described in Methods. Results were normalized by mean densitometric level of time 0 to 100%. Shown is the mean \pm SD (n = 3). * p < .01 vs. time 0. The result is representative of three experiments with similar results.

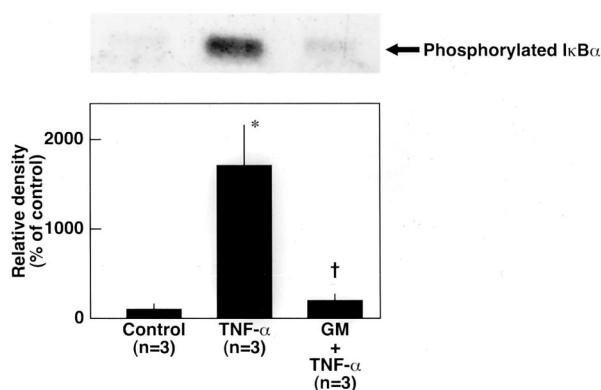


Figure 10. Effect of gabexate mesilate (GM) on tumor necrosis factor (TNF)- α -induced phosphorylation of inhibitor of nuclear factor- κ B ($I\kappa B\alpha$) in human umbilical vein endothelial cells (HUVECs). HUVECs pretreated for 30 mins with or without GM (1.0×10^{-4} M) were incubated for 15 mins with TNF- α (10 units/mL). Whole cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and analyzed for phosphorylated $I\kappa B\alpha$ levels by Western blot analysis as described in Methods. Results were normalized by mean densitometric level of control (without TNF- α stimulation) to 100%. Shown is the mean \pm SD (n = 3). * p < .01 vs. control, † p < .01 vs. TNF- α . The result is representative of three experiments with similar results.

HUVECs by inhibiting degradation of $I\kappa B$ (23). However, GM did not increase the intracellular level of phosphorylated $I\kappa B\alpha$ in HUVECs stimulated with TNF- α but inhibited the TNF- α -induced increase in the intracellular level of phosphorylated $I\kappa B\alpha$, suggesting that GM might inhibit the degradation of $I\kappa B$ by inhibiting phosphorylation of $I\kappa B$. Thus, GM may directly inhibit $I\kappa B$ kinase or some molecular events prerequisite to activation of $I\kappa B$ kinase. Consistent with this hypothesis are the findings of Chen et al. (24) demonstrating that tosyl-lysine-chloromethyl ketone and 3,4-dichloroisocoumarin, serine protease inhibitors, could inhibit

$I\kappa B$ phosphorylation by their alkylating activities. However, since GM is not an alkylating agent, but a competitive inhibitor of serine proteases, mechanisms other than alkylation of $I\kappa B$ kinase might be responsible for inhibition of $I\kappa B$ kinase activity by GM. Further studies are necessary to disclose the mechanisms by which GM inhibited the $I\kappa B$ phosphorylation.

GM competitively inhibits activities of thrombin and factor Xa (25). GM has been used to treat patients with DIC associated with sepsis due to its anticoagulant properties (13). Since the biological half-life of GM in human plasma has been reported to be 55 secs (26), GM is given to

G abexate mesilate inhibited the expression of leukocyte adhesion molecules by inhibiting the nuclear factor- κ B-mediated transcription in human umbilical vein endothelial cells.

patients by continuous intravenous infusion. Plasma anticoagulant activity during the infusion of GM cannot be monitored due to its short-half life in patients (26). Anticoagulant activities of GM are less potent than those of unfractionated heparin, low molecular weight heparin, and heparan sulfate (27): Ki value of GM for thrombin is 1.1×10^{-6} M and that of heparin-antithrombin complex is 2.5×10^{-7} M (25, 27). However, since anticoagulant activities of GM are independent on the presence of antithrombin (13), therapeutic effects of GM could be expected in patients with sepsis whose plasma concentrations of antithrombin are markedly decreased.

We previously reported that GM reduced LPS-induced pulmonary vascular injury by inhibiting TNF- α production in rats (14). Since TNF- α is important for activation of the coagulation system (2), inhibition of TNF- α production by GM might contribute to reduction of the coagulation abnormalities in patients with sepsis. TNF- α also plays an important role in inducing pulmonary vascular injury by activating neutrophils and endothelial cells (3). Anti-E-selectin antibody was shown to reduce *Pseudomonas aeruginosa*-induced lung injury in a porcine sepsis model (28). Gardinali et al. (29) demonstrated that inhibition of binding of the CD11/CD18 complex to ICAM-1 prevented acute lung injury in rabbits with experimental peritonitis. These observations strongly suggest that increases in the expression of E-selectin and ICAM-1 might play a role in the development of sepsis-mediated lung injury. Since the intercellular clefts between tightly adherent activated neutrophils and the endothelium form a microenvironment protected from circu-

lating antiproteases and antioxidants, the neutrophil-endothelial cell interaction is an important aspect of activated neutrophil-induced endothelial cell damage (4, 5). Thus, inhibition of the increases in endothelial leukocyte adhesion molecules by GM might at least partly contribute to the therapeutic effect for LPS-induced pulmonary vascular injury. We also reported that GM reduced the compression-induced spinal cord injury and ischemia/reperfusion-induced liver injury in rats by inhibiting TNF- α production (30, 31). Inhibition of the expression of endothelial leukocyte adhesion molecules by GM also might contribute to these therapeutic effects.

GM is now available for treatment of DIC in patients with sepsis as described previously (13). TNF- α plays an important role in the development of ARDS by activating both neutrophils and endothelial cells (10). ARDS has been shown to adversely affect the outcome of patients with sepsis (15). Although GM has been shown to be effective for reducing pulmonary vascular injury in rats (14), the therapeutic effect for ARDS has not been examined. Clinical trials to evaluate the therapeutic effect of GM should be conducted in patients with sepsis in the near future.

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