

MOL 17442

**Glabridin suppresses intercellular adhesion molecule-1 expression
in TNF- α -stimulated human umbilical vein endothelial cells by
blocking sphingosine kinase pathway: implications of Akt, ERK and
NF- κ B/Rel signaling pathways**

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MOL 17442

Running title: Glabridin inhibits TNF- α -induced gene expression of ICAM-1

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Number of text pages: 43

Number of tables: 0

Number of figures: 11

Number of references: 40

Number of words in Abstract: 239

Number of words in Introduction: 522

Number of words in Discussion: 1492

ABBREVIATIONS: PD98058, 2'-amino-3'methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; SP600125, anthrax[1,9-*cd*]pyrazol-6(2*H*)-one; SKI, 2-(*p*-Hydroxyanilino)-4-(*p*-chlorophenyl) thiazole

MOL 17442

Abstract

(*R*)-4-(3,4-Dihydro-8,8-dimethyl)-2*H*,8*H*-benzo[1,2-*b*:3,4-*b'*]dipyran-3yl)-1,3-benzenediol (glabridin) is known to have anti-inflammatory, antimicrobial and cardiovascular protective activities. In the present study, we report the inhibitory effect of glabridin on intercellular adhesion molecule-1 (ICAM-1) expression in tumor necrosis factor- α (TNF- α)-stimulated human umbilical vein endothelial cells (HUVECs). Glabridin inhibited THP-1 cell adhesion to HUVECs stimulated by TNF- α and cell surface expression of ICAM-1 in TNF- α -stimulated HUVECs. The mRNA expression of adhesion molecules, including ICAM-1, vascular cell adhesion molecule-1 and E-selectin, was also suppressed by glabridin. Further study demonstrated the inhibitory effect of glabridin on nuclear factor (NF)- κ B/Rel DNA binding, inhibitory factor- κ B α (I κ B α) and I κ B β degradation, I κ B kinase activation and p65 nuclear translocation in TNF- α -stimulated HUVECs. Treatment of a variety of cell lines with glabridin revealed that inhibitory effect of glabridin on NF- κ B/Rel activation is not cell type-specific and both inducible and constitutive NF- κ B/Rel activation was suppressed by glabridin treatment. Moreover, TNF- α -induced phosphorylation of Akt and extracellular signal-regulated kinase (ERK) was blocked by glabridin treatment in HUVECs. Glabridin also suppressed sphingosine-1-phosphate (S1P)-induced cell surface expression and mRNA expression of ICAM-1. Further study demonstrated that TNF- α -induced sphingosine kinase activity was

MOL 17442

inhibited by glabridin and the inhibitory effect of glabridin on TNF- α -induced ICAM-1 expression was reversed by addition of exogenous S1P. Collectively, our results indicate that the inhibitory effect of glabridin on ICAM-1 expression might be mediated, at least in part, by inhibiting sphingosine kinase pathway and subsequent inhibition of signaling pathways, including Akt, ERK and NF- κ B/Rel signaling pathway.

MOL 17442

Introduction

Atherosclerosis was formerly considered as a bland lipid storage disease, but it is now regarded as a chronic inflammatory disease (Glass and Witztum, 2001). Recent advances in basic and experimental science have established a fundamental role for inflammation in mediating all stages of this disease from initiation through progression and, ultimately, the thrombotic complications (Blankenberg et al., 2003). The recruitment of inflammatory cells from the circulation and their transendothelial migration are key events in the early phase of atherosclerosis (Glass and Witztum, 2001). Adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), platelet endothelial cellular adhesion molecule-1, E-selectin, P-selectin and L-selectin, are expressed on endothelial cells in response to several inflammatory stimuli and contribute to the recruitment of inflammatory cells to arterial wall and their transmigration across the wall (Blankenberg et al., 2003). ICAM-1, a membrane glycoprotein which belongs to the immunoglobulin superfamily, is widely expressed at a basal level and can be upregulated by proinflammatory cytokines in leukocytes and endothelial cells (Leeuwenberg et al., 1992). ICAM-1 molecules mediate adhesion of leukocytes to activated endothelium by establishing strong bonds with its ligands, leukocyte specific $\beta 2$ integrins (LFA-1, $\alpha L\beta 2$ integrin and etc.), and inducing firm arrest of inflammatory cells at the vascular surface (Chuang et al., 2004). Moreover, Collins and coworkers

MOL 17442

demonstrated that gene deletion of ICAM-1 resulted in significant reductions in monocyte recruitment to atherosclerotic lesions and protection against atherosclerosis in apo E-deficient mice (Collins et al., 2000), suggesting the importance of ICAM-1 in the development of atherosclerosis.

The root of *Glycyrrhiza glabra* (licorice) has been used for centuries as antidotes, demulcents, expectorants and remedies for allergic inflammation, as well as flavoring and sweetening agents in Asia and Europe (Belinky et al., 1998). Glabridin ((*R*)-4-(3,4-Dihydro-8,8-dimethyl)-2*H*,8*H*-benzo[1,2-*b*:3,4-*b'*]dipyran-3yl)-1,3-benzenediol) is a polyphenolic flavonoid and a main constituent in the hydrophobic fraction of licorice extract. It has a wide range of biological activities, including antimicrobial, anti-inflammatory, antinephritic and cardiovascular protective activities (Fukai et al., 2003; Kang et al., 2004; Zhou et al., 2004). Moreover, Fuhrman and coworkers reported that dietary supplementation with licorice extract as well as glabridin results in a substantial reduction in the atherosclerotic lesion area in apoE-deficient mice (Fuhrman et al., 1997) and exhibits anti-atherosclerotic effects in hypercholesterolemic patients (Fuhrman et al., 2002). Recently, it was also reported that glabridin has a protective effect on low density lipoprotein (LDL) oxidation and this contributes to the anti-atherosclerotic effect of glabridin (Belinky et al., 1998; Vaya et al., 1997).

MOL 17442

In the present study, we assessed the effect of glabridin on ICAM-1 expression in human umbilical vein endothelial cells (HUVECs) to further characterize the mechanism involved in the anti-atherosclerotic effect of glabridin. We also investigated the molecular mechanism responsible for the inhibitory effect of glabridin on ICAM-1 gene expression. Here, we showed that glabridin inhibits tumor necrosis factor- α (TNF- α)-induced ICAM-1 expression by blocking sphingosine kinase activity and sphingosine-1-phosphate (S1P) generation. We also demonstrated that the inhibition of S1P signaling by glabridin might be involved in the inhibitory effect of this agent on the activity of Akt, extracellular signal-regulated kinase (ERK) and nuclear factor (NF)- κ B/Rel, and consequently on the ICAM-1 expression in TNF- α -stimulated HUVECs.

MOL 17442

Materials and Methods

Chemicals, animals, and cell culture. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Glabridin was purchased from Wako Pure Chemical Industries, Ltd. (Japan), and dissolved in dimethyl sulfoxide (DMSO) and freshly diluted in culture media for all experiments. S1P was purchased from Biomol (Plymouth meeting, PA, USA), and dissolved in 4 mg/ml of fatty acid free bovine serum albumin (BSA) solution according to manufacturer's instruction. PD98059, SB203580, SP600125 and 2-(*p*-Hydroxyanilino)-4-(*p*-chlorophenyl) thiazole were purchased from Calbiochem (San Diego, CA, USA). Recombinant human TNF- α was purchased from R&D Systems (Minneapolis, MN, USA). The human umbilical vein endothelial cells (HUVECs) were grown in EGM-2® Bulletkit® (Clonetics, BioWhittaker, Inc., MD, USA) and THP-1, U937, A549 and U266 cells were grown in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in 5% CO₂ humidified air.

Cell adhesion assay. Assay was performed as described previously (Oh et al., 2001). Briefly, HUVECs were plated in 48-well plate, grown to 70% confluence and pretreated with glabridin for 1 h, followed by stimulation with TNF- α (10 ng/ml) for 24 h. The cells were rinsed three times with serum free medium, and THP-1 cells were added to each well.

MOL 17442

After 1 h incubation, the non-adherent THP-1 cells were rinsed off and the adherent cells were collected by treatment with Trypsin-EDTA for 1 min and counted directly under a light microscope.

Measurement of ICAM-1 expression. The cell surface expression of ICAM-1 was quantified using a modified enzyme-linked immunosorbent assay (ELISA) according to the method of Gupta and Ghosh with slight modification (Gupta and Ghosh, 1999). Briefly, HUVECs were grown to 70% confluence in 96-well, gelatin-coated plates and treated with glabridin for 1 h before being treated with TNF- α (10 ng/ml) for 24 h. Following incubation, the cells were washed with phosphate-buffered saline, pH 7.4 (PBS) and fixed with 4% paraformaldehyde for 30 min at 4°C. Non-fat dry milk (3.0% in PBS) was added to the monolayers to reduce non-specific binding. After washing three times with PBS, cells were incubated with anti-ICAM-1 monoclonal antibody, washed with PBS and followed by incubation with peroxidase-conjugated goat anti-mouse secondary antibody. Following this, the cells were washed with PBS and exposed to the peroxidase substrate. Absorbance was determined at 450 nm by an automated microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR).

The expressions of the mRNA transcripts of ICAM-1 (forward primer: 5'-CAGTGACCATCTACAGCTTTCCGG -3', reverse primer: 5'-

MOL 17442

GCTGCTACCACAGTGATGATGACAA -3'), VCAM-1 (forward primer: 5'-ATGACATGCTTGAGCCAGG -3', reverse primer: 5'-GTGTCTCCTTCTTTGACACT -3'), E-selectin (forward primer: 5'-GATGTGGGCATGTGGAATGATG -3', reverse primer: 5'-AGGTACACTGAAGGCTCTGG -3'), and β -actin (forward primer: 5'-TGAATCCTGTGGCATCCATGAAAC -3', reverse primer: 5'-TAAAACGCAGCTCAGTAACAGTCCG -3') were evaluated by RT-PCR as described previously (Kang et al., 2003). Briefly, total RNA was isolated using Tri Reagent (Molecular Research Center, Cincinnati, OH, USA) as described previously (Chomczynski and Mackey, 1995). Equal amounts of RNA were reverse transcribed into cDNA using oligo(dT)₁₆ primers. Samples were heated to 94°C for 5 min and cycled 27 times at, 94°C for 30 s, and 60°C for 30 s, and 72°C for 45 s, and this was followed by an additional extension step at 72°C for 5 min. For quantitation of ICAM-1 cDNA, we generated an internal standard as described previously with slight modifications (Kang et al., 2002). DNA fragment for internal standard which can be amplified using ICAM-1 primers listed above and distinguished from wild type PCR products by size, were generated and subcloned into pGEM T Easy vector (Promega Corporation, Madison, WI, USA). The linear relationship of PCR reaction between ICAM-1 cDNA and internal standard was validated and shown in Supplementary figure 1. PCR products were electrophoresed in 3% NuSieve 3:1 gels (FMC Bioproducts, Rockland, ME, USA) and followed by ethidium bromide staining and photography. Band intensities were

MOL 17442

quantified using Image-Quant software (Molecular Dynamics, Sunnyvale, CA, USA).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared as described previously (Kang et al., 2003). The protein content of the nuclear extracts was determined using a Bio-Rad protein assay kit according to the manufacturer's instruction (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The oligonucleotide sequence for NF- κ B/Rel was 5'-GATCTCAGAGGGGACTTTCCGAGAGA-3' (Kang et al., 2003). Double-stranded oligonucleotides were end-labeled with [γ - 32 P]-ATP. Nuclear extracts (5 μ g) were incubated with 2 μ g of poly(dI-dC) and a 32 P-labeled DNA probe, and DNA binding activity was analyzed using a 5% polyacrylamide gel. After electrophoresis, the gel was dried and subjected to autoradiography. The specificity of binding was examined by competition with an unlabeled oligonucleotide. Antibodies for supershift assay (p65, p50, c-Rel and control IgG) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Western immunoblot analysis. 20 μ g of whole cell lysate (for p-Akt, Akt, p-ERK, ERK, p-SAPK/JNK, SAPK/JNK, p-p38 and p38), cytosolic extract (for I κ B α , I κ B β and β -actin) and nuclear extract (p65) were separated by 10% SDS-polyacrylamide gel electrophoresis, and electrotransferred to nitrocellulose membrane (Amersham Bioscience UK, Ltd., Little Chalfont, Buckinghamshire, UK). The membranes were

MOL 17442

preincubated for 1 h at room temperature in Tris-buffered saline, pH 7.6, containing 0.05% Tween 20 and 5% non-fat milk. The nitrocellulose membranes were then incubated with specific antibodies against p-Akt, Akt, p-ERK, ERK, p-SAPK/JNK, SAPK/JNK, p-p38, p38, I κ B α , I κ B β , β -actin, or p65 (Cell Signaling Technology, Beverly, MA, USA). Immunoreactive bands were then detected by incubating with conjugates of anti-rabbit IgG with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham Biosciences UK, Ltd.).

I κ B kinase (IKK) assay. IKK assay was performed as described previously (Lee and Jeon, 2001) with slight modifications. Immunoprecipitation was carried out using anti-IKK α antibody (Cell Signaling Technology) and followed by the kinase assay. The kinase reaction was performed in 30 μ l of kinase buffer (20 mM HEPES, pH 7.8, 10 mM MgCl₂, 100 mM Na₃VO₄, 20 mM β -glycerophosphate, 2 mM dithiothreitol, 50 mM NaCl) for 30 min at 30°C in the presence of 10 mM ATP/10 μ Ci of [γ -³²P]ATP (10 Ci/mmol) and 500 ng of the substrate GST-I κ B α (Santa Cruz Biotechnology). The reactions were terminated with 4X sample buffer. Proteins were analyzed on 12% SDS-polyacrylamide gels, dried and visualized by autoradiography. Direct effect of glabridin on IKK α activity was assessed using constitutively active recombinant IKK β (Upstate, Charlottesville, VA, USA) rather than using immunoprecipitated IKK.

Sphingosine kinase assay. Sphingosine kinase assay was performed according to the method of Edsall and coworkers with slight modifications (Edsall et al., 1997). After various treatments, cells were washed twice with PBS and harvested by scraping in buffer A (0.1 M Tris-HCl, pH 7.4, containing 20% (v/v) glycerol, 1 mM mercaptoethanol, 1 mM EDTA, 1 mM Na₃VO₄, 15 mM NaF, 10 µg/ml leupeptin and aprotinin, 1 mM PMSF, and 0.5 mM 4-deoxypyridoxine). Cells were lysed by freeze-thawing three times, and the cytosolic fraction was prepared by centrifugation at 13,000 X g for 20 min. 10 µl of sphingosine (1 mM), delivered as a sphingosine-BSA complex (in 4 mg/ml BSA) was added to 100 µl of cytosolic fraction (50 µg), reaction volume was adjusted to 190 µl with buffer A, and reactions were started by addition of 10 µl of [γ -³²P]ATP (10 µCi, 20 mM) containing 100 mM MgCl₂. Samples were incubated for 30 min at 37°C. Lipids were then extracted with chloroform/methanol/concentrated HCl (100:200:1, v/v), and phases were separated. Lipids from the organic phase were resolved by TLC on Silica Gel 60 plates using 1-butanol/methanol/acetic acid/ water (80:20:10:20, v/v) as the solvent system. Labeled sphingosines were visualized by autoradiography, scraped from the plate, and counted with a scintillation counter.

Statistical analysis. The mean \pm S.D. was determined for each treatment group in each experiment. Data were analyzed by analysis of

MOL 17442

variance (ANOVA), and Student's *t* test was used for comparisons of multiple comparisons. Differences were considered significant if *p* values are less than or equal to 0.05.

MOL 17442

Results

Inhibitory effect of glabridin on THP-1 cell adhesion to HUVECs stimulated with TNF- α . As described previously, monocyte adhesion to endothelial cells is an important event in the initiation of atherosclerosis development. Therefore, we examined the effect of glabridin on THP-1 cell adhesion to TNF- α -stimulated HUVECs to assess the mechanism responsible for the anti-atherosclerotic effect of glabridin. When unstimulated, the binding of THP-1 cells to HUVECs was at the basal level. However, THP-1 cell adhesion was substantially increased when HUVECs were stimulated with TNF- α (10 ng/ml) (Fig. 1), and the increased adhesion of THP-1 cells to TNF- α -stimulated HUVECs was significantly suppressed by glabridin in a dose-dependent manner (Fig. 1). As shown in Fig. 1, treatment with 3 μ M and 10 μ M of glabridin caused 46 and 65% inhibition of THP-1 cell adhesion to TNF- α -stimulated HUVECs, respectively. The concentration and duration of glabridin treatment used in this study had no significant effect on the viability of HUVECs (Fig. 2A).

Glabridin suppressed the cell surface expression of ICAM-1 in TNF- α -stimulated HUVECs. Adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin, are involved in the adhesion of inflammatory cells to endothelium (Glass and Witztum, 2001). Among these adhesion molecules, ICAM-1 is known to promote adhesion of monocytes,

MOL 17442

neutrophils, and lymphocytes to endothelium and has been detected in atherosclerotic lesions in humans (Poston et al., 1992). We also previously reported that functional blocking of ICAM-1 with anti-ICAM-1 antibody reduced THP-1 cell adhesion to TNF- α -stimulated HUVECs (Kang et al., 2003). Therefore, we focused on the inhibitory effect of glabridin on ICAM-1 in this report. Here, we examined the effect of glabridin on the cell surface expression of ICAM-1 in TNF- α -stimulated HUVECs to further characterize the reason why glabridin inhibited THP-1 cell adhesion to TNF- α -stimulated HUVECs. As shown in Fig. 2B, TNF- α (10 ng/ml) alone increased ICAM-1 expression by 3.8-fold in HUVECs, and this induction of ICAM-1 expression was concentration dependently suppressed by glabridin. Treatment of TNF- α -stimulated HUVECs with 3 μ M and 10 μ M of glabridin caused 57% and 69% inhibition of the cell surface expression of ICAM-1 (Fig. 2B).

Effect of glabridin on the mRNA expression of ICAM-1 and other adhesion molecules in TNF- α -stimulated HUVECs. To investigate whether the inhibition of TNF- α -induced cell surface expression of ICAM-1 by glabridin is mediated by suppression of ICAM-1 gene expression, we assessed the effect of glabridin on ICAM-1 mRNA expression in TNF- α -stimulated HUVECs by RT-PCR. The level of ICAM-1 mRNA expression was markedly increased after stimulation with TNF- α (10 ng/ml) (Fig. 3A). Consistent with previous results, TNF- α -induced expression of ICAM-1 mRNA was significantly suppressed by glabridin in

MOL 17442

a dose-dependent manner (Fig. 3A). Additionally, we assessed the mRNA expression of other adhesion molecules, such as VCAM-1 and E-selectin. As shown in Fig. 3B, TNF- α -induced mRNA expression of VCAM-1 and E-selectin was also inhibited by glabridin. The mRNA expression of β -actin was not significantly affected by either TNF- α or glabridin (Fig. 3B).

Inhibition of NF- κ B/Rel DNA binding by glabridin in TNF- α -stimulated HUVECs. NF- κ B/Rel is known to have a critical role in the expression of proinflammatory adhesion molecules, and also shown to be important for ICAM-1 expression (Ledebur and Parks, 1995). To further investigate the mechanism involved in the inhibitory effect of glabridin on ICAM-1 gene expression, we assessed the effect of glabridin on NF- κ B/Rel DNA binding in TNF- α -stimulated HUVECs. Treatment of HUVECs with TNF- α (10 ng/ml) caused a significant increase in NF- κ B/Rel DNA binding within 30 min. In the presence of glabridin, TNF- α -induced DNA binding of NF- κ B/Rel was suppressed in a concentration dependent manner (Fig. 4A). To investigate whether glabridin inhibits NF- κ B/Rel DNA binding by directly modifying NF- κ B/Rel proteins, we incubated nuclear extracts of TNF- α -treated cells with glabridin for 1 h at room temperature. After incubation, DNA binding was determined using electrophoretic mobility shift assay. Fig. 4B shows that glabridin did not directly modify the DNA binding ability of NF- κ B/Rel proteins. Supershift analysis using antibodies directed against p65, p50 and c-Rel revealed

MOL 17442

that the main components of NF- κ B/Rel DNA binding in TNF- α -stimulated HUVECs were p65 and p50. Isotype matched irrelevant control antibody had no effect on NF- κ B/Rel DNA binding. The specificity of DNA binding was also confirmed using unlabeled NF- κ B/Rel oligonucleotide and mutant NF- κ B/Rel oligonucleotide (Fig. 4C).

Effect of glabridin on TNF- α -induced NF- κ B/Rel DNA binding in various cell types. To investigate cell type specificity of the inhibitory effect of glabridin on NF- κ B/Rel activation, we assessed the effect of glabridin on TNF- α -induced increase in NF- κ B/Rel DNA binding in human histiocytic lymphoma (U937), human lung epithelial cell carcinoma (A549) cells. As shown in Fig. 4D, glabridin suppressed the induction of NF- κ B/Rel DNA binding in both cell types stimulated with TNF- α . Next, we examined the effect of glabridin on the NF- κ B/Rel DNA binding in human multiple myeloma (U266) cells in which NF- κ B/Rel is constitutively active. Glabridin treatment also down-regulated this constitutively active NF- κ B/Rel DNA binding in U266 cells (Fig. 4D).

Effect of glabridin on I κ B degradation, IKK activation and p65 nuclear translocation in TNF- α -stimulated HUVECs. The activation of NF- κ B/Rel occurs *via* nuclear translocation of Rel family proteins and this is preceded by the phosphorylation and degradation of I κ Bs by IKK. Therefore, to further investigate the mechanism responsible for the inhibitory effect of glabridin on NF- κ B/Rel activation, we examined the

MOL 17442

effect of glabridin on the degradation of I κ B α and I κ B β using Western immunoblot. As shown in Fig. 5A, I κ B α was degraded by TNF- α (10 ng/ml) treatment within 10 min, rapidly resynthesized and returned to normal level with 60 min. In contrast to I κ B α , the degradation and recovery of I κ B β was delayed and the level of I κ B β protein was low until 60 min. The degradation of both I κ B α and I κ B β was blocked by glabridin (10 μ M) in TNF- α -stimulated HUVECs (Fig. 5A). The level of β -actin was unaffected by either TNF- α or glabridin treatment (Fig. 5A). Next, we examined the effect of glabridin on IKK activity in TNF- α -stimulated HUVECs. Fig. 5B shows that glabridin suppressed TNF- α -induced activation of IKK activity. We also assessed direct effect of glabridin on the activity of IKK β using constitutively active recombinant IKK β . As shown in Fig. 5B, glabridin had no direct effect on IKK β activity. To further investigate, we assessed the effect of glabridin on the nuclear translocation of NF- κ B/Rel p65 subunit. Consistent with previous results, p65 nuclear translocation was also suppressed by glabridin treatment (Fig. 5C).

Glabridin inhibits Akt activity in TNF- α -stimulated HUVECs.

Akt is implicated in the regulation of NF- κ B/Rel activity (Gustin et al., 2004). Moreover, TNF- α has been shown to activate NF- κ B/Rel through activation of Akt (Ozes et al., 1999). Therefore, we examined the effect of glabridin on TNF- α -induced Akt activation in HUVECs. As shown in Fig. 6A, TNF- α induced Akt phosphorylation and the increase in Akt

MOL 17442

phosphorylation by TNF- α was concentration-dependently suppressed by glabridin treatment. To investigate the relation between Akt activation and ICAM-1 expression, we examined the effect of LY294002 and wortmanin, specific inhibitors of phosphatidylinositol 3-kinase (PI3K)/Akt pathway, on the expression of ICAM-1 in TNF- α -stimulated HUVECs. Our data shows that the inhibition of Akt activation resulted in the suppression of ICAM-1 expression (Fig. 6B). Moreover, involvement of Akt in the regulation of NF- κ B/Rel activation was also confirmed. As shown in Fig. 6C, LY294002 and wortmanin suppressed TNF- α -induced NF- κ B/Rel DNA binding in HUVECs.

Effect of glabridin on the activity of mitogen-activated protein kinases (MAPKs) in TNF- α -stimulated HUVECs. MAPK is known to regulate the gene expression of ICAM-1 and the activity of NF- κ B/Rel. To further characterize the mechanism responsible for the inhibitory effect of glabridin on ICAM-1 expression and NF- κ B/Rel activity, we examined the effect of glabridin on MAPK activation using Western immunoblot analysis. As shown in Fig. 7A, TNF- α -induced phosphorylation of extracellular signal-regulated kinase (ERK) was dose-dependently suppressed to basal level by glabridin treatment in HUVECs. However, the effect of glabridin on stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and p38 MAP kinase was weak, and the activations of these kinases were slightly inhibited by highest dose (10 μ M) of glabridin (Fig. 7A). We also assessed the effect of MAPK

MOL 17442

inhibitors on the expression of ICAM-1 in TNF- α -stimulated HUVECs. Fig. 7B shows that PD98059, an inhibitor of ERK, significantly suppressed ICAM-1 expression while SP600125 and SB203580, SAPK/JNK and p38 MAP kinase inhibitor, respectively, had no significant effect. The effect of MAPK inhibitors on NF- κ B/Rel activation was also tested. PD98059 suppressed TNF- α -induced NF- κ B/Rel DNA binding in HUVECs as demonstrated in Fig. 7C. However, SP600125 and SB203580 had little effect on NF- κ B/Rel DNA binding in TNF- α -stimulated HUVECs (Fig. 7C).

Inhibition of S1P-induced expression of ICAM-1 and activation of Akt, ERK and NF- κ B/Rel by glabridin in HUVECs. It has been known that TNF- α induces endothelial cell activation and adhesion molecule expression by stimulation of S1P generation and S1P alone can induce expression of ICAM-1 and activation of Akt, ERK and NF- κ B/Rel in HUVECs (Baudhuin et al., 2002; Xia et al., 1998). Here, we investigated the effect of glabridin on S1P-induced expression of ICAM-1 and activation of various signaling molecules to investigate whether the inhibitory effect of glabridin on TNF- α -induced ICAM-1 expression is mediated by the inhibition of S1P signaling pathway. First, we confirmed the induction of cell surface expression and mRNA expression of ICAM-1 by S1P. Fig. 8A shows that 5 μ M of S1P significantly increased cell surface expression of ICAM-1 in HUVECs. ICAM-1 mRNA expression was also induced by S1P treatment in a dose-related manner (Fig. 8B). However, S1P-induced cell surface expression and mRNA expression of

MOL 17442

ICAM-1 was suppressed by glabridin (10 μ M) treatment (Fig. 8C and Fig. 8D). Because it has been reported that S1P-induced expression of adhesion molecules is mediated by Akt, ERK and NF- κ B/Rel signaling pathway (Baudhuin et al., 2004; Xia et al., 1998), we also examined the effect of glabridin on the activation of these pathways induced by S1P. As shown in Fig. 8E, the phosphorylation of Akt and ERK was increased by S1P (5 μ M) treatment. However, S1P-mediated increase in Akt and ERK phosphorylation was completely blocked by glabridin (10 μ M) treatment (Fig. 8E). In addition, NF- κ B/Rel DNA binding was increased by S1P (5 μ M) treatment, and this was also suppressed by glabridin (10 μ M) treatment (Fig. 8F).

Inhibition of TNF- α -induced ICAM-1 expression by a specific inhibitor of sphingosine kinase. To further confirm whether sphingosine kinase signaling pathway is involved in TNF- α -induced expression of ICAM-1, we examined the effect of SKI (2-(*p*-Hydroxyanilino)-4-(*p*-chlorophenyl) thiazole) on TNF- α -induced ICAM-1 expression. As shown in Fig. 9A, SKI significantly inhibited TNF- α -induced cell surface expression of ICAM-1 in HUVECs. TNF- α -induced mRNA expression of ICAM-1 was also significantly suppressed by SKI treatment in HUVECs (Fig. 9B). SKI alone had no significant effect on cell surface expression and mRNA expression of ICAM-1 in HUVECs (Fig. 9A and Fig. 9B).

Inhibition of sphingosine kinase activity by glabridin in TNF- α -stimulated HUVECs. To further investigate the effect of glabridin on sphingosine kinase signaling pathway, we examined the effect of glabridin on sphingosine kinase activity in TNF- α -stimulated HUVECs. As shown in Fig. 10A, TNF- α (10 ng/ml) caused 2.0-fold increase in sphingosine kinase activity in HUVECs, and glabridin significantly suppressed TNF- α -induced sphingosine kinase activity in HUVECs. Glabridin alone slightly inhibited the basal level of sphingosine kinase activity, but the effect was not significant (Fig. 10A).

Reversal of the inhibitory effect of glabridin on TNF- α -induced ICAM-1 expression by addition of exogenous S1P. To further confirm whether the inhibitory effect of glabridin on TNF- α -induced ICAM-1 expression was mediated through the suppression of S1P generation, we examined the effect of exogenous S1P addition on the inhibition of ICAM-1 expression by glabridin. The results in Fig. 10B and Fig. 10C show that the suppression of cell surface expression and mRNA expression of ICAM-1 was reversed by the addition of S1P.

MOL 17442

Discussion

Licorice extract as well as glabridin are known to have an anti-atherosclerotic activity in apoE-deficient mice and hypercholesterolemic patients (Fuhrman et al., 1997; Fuhrman et al., 2002), and it was reported that the anti-atherosclerotic activity of glabridin is mediated by its protective effect on LDL oxidation (Belinky et al., 1998; Vaya et al., 1997). Here, we hypothesized that the expression of inflammatory adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin may be affected by glabridin. In the present study, we clearly demonstrated that glabridin inhibits THP-1 cell adhesion to HUVECs stimulated with TNF- α and ICAM-1 expression in TNF- α -stimulated HUVECs. The mRNA expression of all of three adhesion molecules tested in this report, including ICAM-1, VCAM-1 and E-selectin, was also down-regulated by glabridin treatment. These adhesion molecules are well known to be important for the attachment of inflammatory cells to arterial wall (Glass and Witztum, 2001), and our previous report also showed that the functional blocking of these adhesion molecules suppressed THP-1 cell adhesion to HUVECs (Kang et al., 2003). Therefore, it is assumed that the inhibition of THP-1 cell adhesion to HUVECs by glabridin is mediated by its inhibitory effect on the adhesion molecule expression in HUVECs. Collectively, the inhibitory effect of glabridin on THP-1 cell adhesion and adhesion molecule expression presented in this report provide a new mechanism responsible for the anti-atherosclerotic activity of glabridin.

NF- κ B/Rel is known as a pleiotropic regulator of various genes involved in immune and inflammatory responses (Ghosh et al., 1998). It has also been reported that transcriptional activation of NF- κ B/Rel is important for the expression of inflammatory adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin (Kang et al., 2003). Therefore, to further investigate the molecular mechanism responsible for the inhibitory effect of glabridin on adhesion molecule expression, we examined the effect of glabridin on NF- κ B/Rel activity. Our results demonstrated that glabridin potently suppressed NF- κ B/Rel DNA binding in TNF- α -stimulated HUVECs. Consistent with previous report (Kim et al., 2004), p65 and p50 were found to be the main components of TNF- α -induced NF- κ B/Rel DNA binding in HUVECs. The assessment of direct effect of glabridin on NF- κ B/Rel DNA binding activity showed that glabridin had no direct effect, suggesting that the inhibition of NF- κ B/Rel activity by glabridin is mediated by modulation of upstream signaling pathway involved in NF- κ B/Rel activation. Further studies revealed that the inhibitory effect of glabridin on NF- κ B/Rel DNA binding was mediated by blocking degradation of I κ B α and I κ B β and nuclear translocation of p65. Glabridin also inhibited the activity of IKK in TNF- α -stimulated HUVECs, but direct addition of glabridin to constitutively active IKK β had no effect on its activity, indicating that glabridin might modulate the signaling pathways regulating IKK activity.

MOL 17442

To further investigate the mechanism responsible for glabridin-mediated suppression of NF- κ B/Rel activation, we examined the effect of glabridin on upstream signaling pathways, such as PI3K/Akt pathway and MAPK pathway. Here, we clearly demonstrated that glabridin inhibited TNF- α -induced activation of Akt and ERK. Akt was reported to be involved in the NF- κ B/Rel activation induced by TNF- α and platelet-derived growth factors (Ozes et al., 1999; Romashkova and Makarov, 1999). Akt transiently associates *in vivo* with IKK and induces IKK activation through IKK phosphorylation at threonine 32 (Ozes et al., 1999; Romashkova and Makarov, 1999). Madrid and coworkers reported that Akt can suppress apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF- κ B/Rel (Madrid et al., 2000). In the present study, we demonstrated that specific inhibitors of PI3K/Akt pathway, LY294002 and wortmannin, inhibit TNF- α -induced NF- κ B/Rel activation in HUVECs, suggesting the involvement of Akt signaling pathway in the regulation of NF- κ B/Rel activity in our system. MAPKs are another intracellular mediators involved in the regulation of NF- κ B/Rel activation. NF- κ B/Rel has been reported to be a downstream target of MEK/ERK pathway (Jiang et al., 2004; Wang et al., 2004). Consistent with these reports, our data also shows that specific inhibitor of MEK/ERK pathway, PD98059, suppressed NF- κ B/Rel activation in TNF- α -stimulated HUVECs, indicating that MEK/ERK pathway is important for NF- κ B/Rel activation in our system. Collectively, our results suggest that Akt and ERK are upstream regulators of NF- κ B/Rel activation in TNF- α -

MOL 17442

stimulated HUVECs and the inhibitory effect of glabridin on NF- κ B/Rel activity is mediated, at least in part, by inhibiting Akt and ERK activation.

S1P is generated by phosphorylation of sphingosine by sphingosine kinase and it has been proposed to act both as an extracellular mediator and as an intracellular second messenger (Fig. 11). Extracellular effect of S1P is mediated via a family of plasma membrane G-protein-coupled receptors, which are now known as S1P receptors. Activation of S1P receptors regulate a variety of intracellular signaling pathways leading to mitogenesis, chemotaxis, differentiation and apoptosis (Pyne and Pyne, 2000). Recently, an intracellular second messenger role for S1P was suggested by demonstrating only a partial inhibition of S1P-induced ERK pathway by pertussis toxin (Wu et al., 1995). S1P has been shown to modulate several signaling cascade, including Ras/Raf/MEK/ERK pathway and NF- κ B/Rel pathway, as a second messenger (Spiegel and Milstien, 2003, Pyne, 2000 #46). Previously, it was reported that the activation of sphingosine kinase and concomitant generation of S1P is critically involved in TNF- α -induced expression of VCAM-1 and E-selectin in HUVECs (Xia et al., 1998). In addition, S1P-mediated induction of MEK/ERK and PI3K/Akt pathway was demonstrated in a variety of cell types, including rat vascular smooth muscle cells, U-373 MG glioblastoma cell line and HEY ovarian cancer cell line (Baudhuin et al., 2002; Tanimoto et al., 2004; Van Brocklyn et al., 2002). Xia and coworkers also showed that S1P can induce ERK and

MOL 17442

NF- κ B/Rel activation in HUVECs (Xia et al., 1998). These reports suggest that S1P is an important mediator of Akt, ERK and NF- κ B/Rel activation. In the present study, we clearly demonstrated that S1P increases cell surface and mRNA expression of ICAM-1 in HUVECs. S1P-induced cell surface and mRNA expression of ICAM-1 was almost completely blocked by glabridin treatment. Moreover, in agreement with previous reports, S1P caused activation of Akt, ERK and NF- κ B/Rel and this was abrogated by glabridin treatment. We also demonstrated the inhibition of sphingosine kinase activity by glabridin and the reversal of the inhibitory effect of glabridin on TNF- α -induced ICAM-1 expression by exogenous addition of S1P, suggesting that glabridin might inhibit TNF- α -induced ICAM-1 expression by blocking sphingosine kinase signaling. One more thing to be considered is that the inhibitory effect of glabridin on NF- κ B/Rel activation might also be mediated by S1P-independent signaling pathways. One of the possible S1P-independent signaling pathway regulating NF- κ B/Rel is reactive oxygen species (ROS) signaling pathway. NF- κ B/Rel itself has been well known to be sensitive to redox status (Kang et al., 2004). Moreover, both Akt and ERK have been known to be regulated by ROS signaling pathway (Knebel et al., 1996; Wang et al., 2000). Corda and coworkers also reported that ROS is rapidly produced in HUVECs in response to TNF- α . Because glabridin has an antioxidant activity, we can speculate that glabridin might also down-regulate NF- κ B/Rel activity by inhibiting ROS-mediated Akt and ERK activation. However, further study is required to prove this

MOL 17442

speculation. Collectively, our results suggest that the suppression of TNF- α -induced ICAM-1 expression by glabridin might be mediated, at least in part, by blocking sphingosine kinase activity and S1P generation.

An oxLDL is implicated in the development of atherosclerosis and the uptake of oxLDL by macrophages leads to the development of atherosclerotic lesion. Moreover, oxLDL itself has inflammatory and atherogenic properties and accelerates the progression of atherosclerosis (Glass and Witztum, 2001). As mentioned earlier, inhibition of LDL oxidation by glabridin was suggested as a possible mechanism responsible for its anti-atherosclerotic activity (Belinky et al., 1998; Vaya et al., 1997). Nitric oxide (NO) exerts critical and diverse functions in the cardiovascular system and an impaired production of NO plays a key role in the development of cardiovascular diseases. NO is known to have both atherogenic and vascular protective effects, depending on the source and the amount of production. NO produced by endothelial NO synthase (NOS) has a vasodilator function and has a protective effect. However, inducible NOS in macrophages produces a large amount of NO in response to stimuli, and potent oxidative properties of NO produced by iNOS appear to induce atherosclerosis (Cooke and Dzau, 1997). Therefore, our previous study showing the inhibitory effect of glabridin on iNOS expression in macrophages also suggest one of the anti-atherosclerotic mechanisms of this agent (Kang et al., 2004). Including the inhibitory effect of glabridin on adhesion

MOL 17442

molecule expression demonstrated in the present study, all of the properties of glabridin mentioned above seem to contribute anti-atherosclerotic activity of glabridin.

In summary, we demonstrated here that glabridin inhibits THP-1 cell adhesion to HUVECs stimulated by TNF- α by suppressing the gene expression of adhesion molecules, especially ICAM-1. Our results also showed that the inhibitory effect of glabridin on adhesion molecule expression is mediated by blocking sphingosine kinase pathway and subsequent inhibition of Akt, ERK and NF- κ B/Rel in HUVECs. The results presented in this report give an insight into the mechanism responsible for the anti-atherosclerotic activity of glabridin and licorice extract.

MOL 17442

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MOL 17442

Footnotes

This research was supported by a grant from KRIBB Research Initiative Program.

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MOL 17442

Legends for figures

Fig. 1. Effect of glabridin on THP-1 cell adhesion to HUVECs stimulated with TNF- α . HUVECs were pretreated with vehicle (DMSO) or indicated concentrations of glabridin for 1 h, followed by stimulation with TNF- α (10 ng/ml) for 24 h. THP-1 cells were added to each well for 1 h, non-adherent cells were rinsed off, adherent cells were collected and counted under a light microscope. Each column shows the mean \pm S.D. of triplicate determinations. Asterisks (*) indicate that *p* values are less than or equal to 0.05.

Fig. 2. Inhibition of cell surface expression of ICAM-1 by glabridin in TNF- α -stimulated HUVECs. (A) HUVECs were treated with vehicle (DMSO) or indicated concentrations of glabridin in the presence or absence of TNF- α (10 ng/ml). 24 h after TNF- α treatment, cell viability was measured by MTT assay. (B) HUVECs were pretreated with vehicle (DMSO) or indicated concentrations of glabridin for 1 h before being incubated with TNF- α (10 ng/ml) for 24 h. Cell-based ELISA was performed as described in Materials and Methods. Each column shows the mean \pm S.D. of triplicate determinations. Asterisks (*) indicate that *p* values are less than or equal to 0.05.

Fig. 3. Inhibition of the mRNA expression of adhesion molecules by

MOL 17442

glabridin in TNF- α -stimulated HUVECs. (A) HUVECs were pretreated with vehicle (DMSO) or indicated concentrations of glabridin for 1 h before being incubated with TNF- α (10 ng/ml) for 6 h. Total RNA was isolated and the mRNA expression of ICAM-1 was quantitated by competitive RT-PCR as described in Materials and Methods. Each column shows the mean \pm S.D. of triplicate determinations. Asterisks (*) indicate that *p* values are less than or equal to 0.05. (B) VCAM-1, E-selectin and β -actin mRNA expression was determined by RT-PCR as described in Materials and Methods.

Fig. 4. Effect of glabridin on NF- κ B/Rel DNA binding. (A) HUVECs were pretreated with vehicle (DMSO) or indicated concentrations of glabridin for 1 h before being incubated with TNF- α (10 ng/ml) for 30 min. Nuclear extracts were prepared and NF- κ B/Rel DNA binding was determined by EMSA as described in Materials and Methods. (B) Nuclear extracts isolated from TNF- α -stimulated HUVECs were incubated with glabridin (1 or 10 μ M) for 1 h and NF- κ B/Rel DNA binding was determined by EMSA. (C) Nuclear extracts isolated from TNF- α -treated HUVECs were incubated with the indicated antibodies (anti-p65 Ab, anti-p50 Ab, anti-cRel Ab and isotype matched control Ab) or oligonucleotides (cold oligonucleotide and mutant cold oligonucleotide) for 30 min and NF- κ B/Rel DNA binding was determined by EMSA. (D) U937 and A549 cells were pretreated with vehicle (DMSO) or glabridin (1 or 10 μ M) for 1 h

MOL 17442

before being incubated with TNF- α (10 ng/ml) for 30 min. U266 cells were treated with glabridin (1 or 10 μ M) for 2 h. Nuclear extracts were prepared and NF- κ B/Rel DNA binding was determined by EMSA.

Fig. 5. Effect of glabridin on I κ B α and I κ B β degradation, IKK activation and p65 nuclear translocation in TNF- α -stimulated HUVECs. (A) HUVECs were pretreated with vehicle (DMSO) or glabridin (10 μ M) for 1 h before being incubated with TNF- α for indicated times. Cytosolic extracts were prepared and the degradation of I κ Bs was determined by Western immunoblot analysis as described in Materials and Methods. (B) HUVECs were pretreated with vehicle (DMSO) or glabridin (10 μ M) for 1 h before being incubated with TNF- α for 30 min. Total cell lysates were prepared and IKK complex was immunoprecipitated using anti-IKK α antibody. Kinase assay was performed as described in Materials and Methods (Upper panel). Recombinant human IKK β was incubated with glabridin (10 μ M) for 10 min and kinase assay was performed (Lower panel). (C) HUVECs were pretreated with vehicle (DMSO) or glabridin (10 μ M) for 1 h before being incubated with TNF- α for 30 min. Nuclear extracts were prepared and the level of p65 was determined by Western Immunoblot analysis.

Fig. 6. Effect of glabridin on Akt activation in TNF- α -stimulated HUVECs. (A) HUVECs were pretreated with vehicle (DMSO) or indicated concentrations of glabridin for 1 h before being incubated with TNF- α for

MOL 17442

30 min. Total cell lysates were prepared and Akt phosphorylation was determined using Western immunoblot analysis. (B) HUVECs were pretreated with vehicle (DMSO), LY (LY294002, 10 μ M), WO (wortmannin, 10 μ M) or Gla (glabridin, 10 μ M) for 1 h before being incubated with TNF- α for 24 h. Cell surface expression of ICAM-1 was determined by cell-based ELISA. Each column shows the mean \pm S.D. of triplicate determinations. Asterisks (*) indicate that *p* values are less than or equal to 0.05. (C) HUVECs were pretreated with vehicle (DMSO), LY (LY294002, 10 μ M) or WO (wortmannin, 10 μ M) for 1 h before being incubated with TNF- α for 30 min. Nuclear extract were prepared and NF- κ B/Rel DNA binding was performed by EMSA.

Fig. 7. Effect of glabridin on MAPKs activation in TNF- α -stimulated HUVECs. (A) HUVECs were pretreated with vehicle (DMSO) or indicated concentrations of glabridin for 1 h before being incubated with TNF- α for 30 min. Total cell lysates were prepared and phosphorylation of ERK, SAPK/JNK and p38 MAP kinase was determined using Western immunoblot analysis. (B) HUVECs were pretreated with vehicle (DMSO), PD (PD98059, 10 μ M), SP (Sp600125, 10 μ M), SB (SB203580, 10 μ M) or Gla (glabridin, 10 μ M) for 1 h before being incubated with TNF- α for 24 h. Cell surface expression of ICAM-1 was determined by cell-based ELISA. Each column shows the mean \pm S.D. of triplicate determinations. Asterisks (*) indicate that *p* values are less than or equal to 0.05. (C)

MOL 17442

HUVECs were pretreated with vehicle (DMSO), PD (PD98059, 10 μ M), SP (Sp600125, 10 μ M) or SB (SB203580, 10 μ M) for 1 h before being incubated with TNF- α for 30 min. Nuclear extracts were prepared and NF- κ B/Rel DNA binding was performed by EMSA.

Fig. 8. Induction of cell surface expression and mRNA expression of ICAM-1 by S1P and inhibition of S1P-induced ICAM-1 expression by glabridin. (A, B) HUVECs were treated with vehicle, indicated concentrations S1P or TNF- α for 24 h (cell surface expression) or 6 h (mRNA expression). Cell surface expression and mRNA expression of ICAM-1 were analyzed by cell-based ELISA and competitive RT-PCR as described in Materials and Methods. (C, D) HUVECs were treated with vehicle (DMSO) or glabridin for 1 h before being incubated with S1P or TNF- α for 24 h (cell surface expression) or 6 h (mRNA expression). Cell surface expression and mRNA expression of ICAM-1 were analyzed by cell-based ELISA and competitive RT-PCR. Each column shows the mean \pm S.D. of triplicate determinations. Asterisks (*) indicate that *p* values are less than or equal to 0.05. (E, F) HUVECs were treated with vehicle (DMSO) or glabridin for 1 h before being incubated with S1P for 30 min. Akt and ERK phosphorylation and NF- κ B/Rel activation was measured by Western immunoblot analysis and EMSA.

Fig. 9. Effect of a specific inhibitor of sphingosine kinase on TNF- α -

MOL 17442

induced cell surface expression and mRNA expression of ICAM-1 in HUVECs. (A) HUVECs were pretreated with SKI (1 μ M) for 1 h before being incubated with TNF- α (10 ng/ml) for 24 h. Cell surface expression of ICAM-1 was analyzed by cell-based ELISA as described in Materials and Methods. (B) HUVECs were pretreated with SKI (1 μ M) for 1 h before being incubated with TNF- α (10 ng/ml) for 6 h. Total RNA was prepared and ICAM-1 mRNA expression was quantitated by competitive RT-PCR. Each column shows the mean \pm S.D. of triplicate determinations. Asterisks (*) indicate that *p* values are less than or equal to 0.05.

Fig. 10. Inhibition of sphingosine kinase activity by glabridin and reversal of inhibitory effect of glabridin on TNF- α -induced ICAM-1 expression by addition of exogenous S1P. (A) HUVECs were pretreated with glabridin (10 μ M) for 1 h before being incubated with TNF- α (10 ng/ml) for 10 min. Cytosolic extracts were prepared and sphingosine kinase activity was measured as described in Materials and Methods. HUVECs were treated with vehicle (DMSO) or glabridin (10 μ M) for 1 h before being incubated with TNF- α for 24 h (cell surface expression) or 6 h (mRNA expression). Cell surface expression (B) and mRNA expression (C) of ICAM-1 was analyzed by cell-based ELISA and competitive RT-PCR. Each column shows the mean \pm S.D. of triplicate determinations. Asterisks (*) indicate that *p* values are less than or equal to 0.05.

MOL 17442

Fig. 11. Schematic diagram of TNF- α -induced signaling pathways in HUVECs and interruption of TNF- α -induced signaling by glabridin and various inhibitors.

Fig. 1.

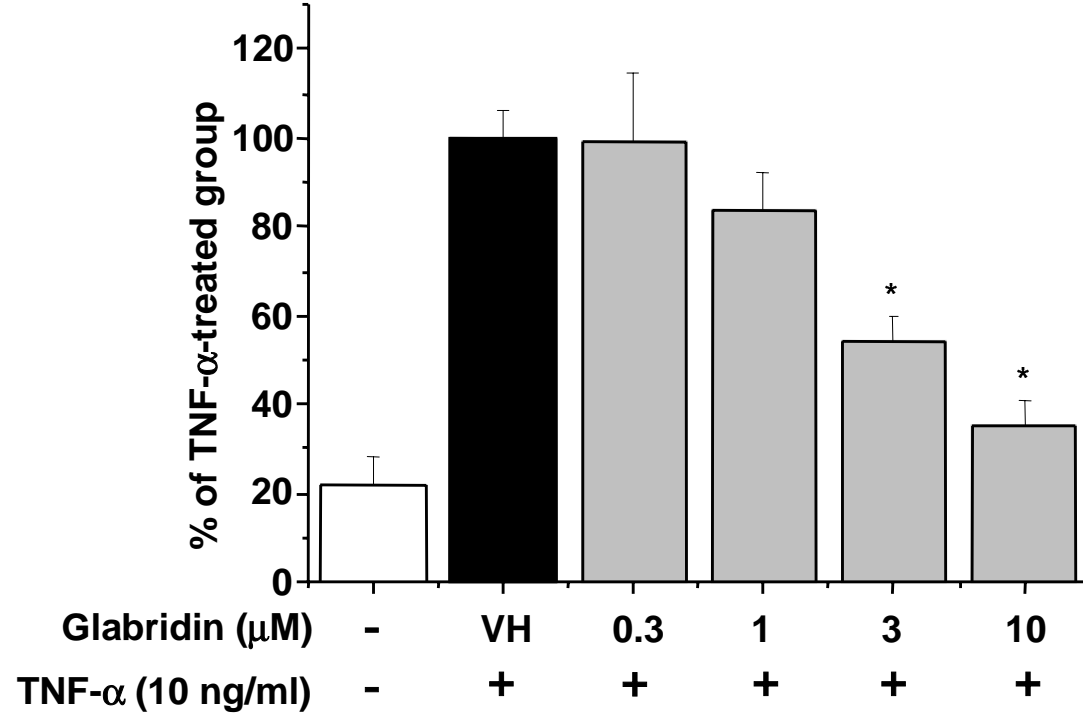


Fig. 2.

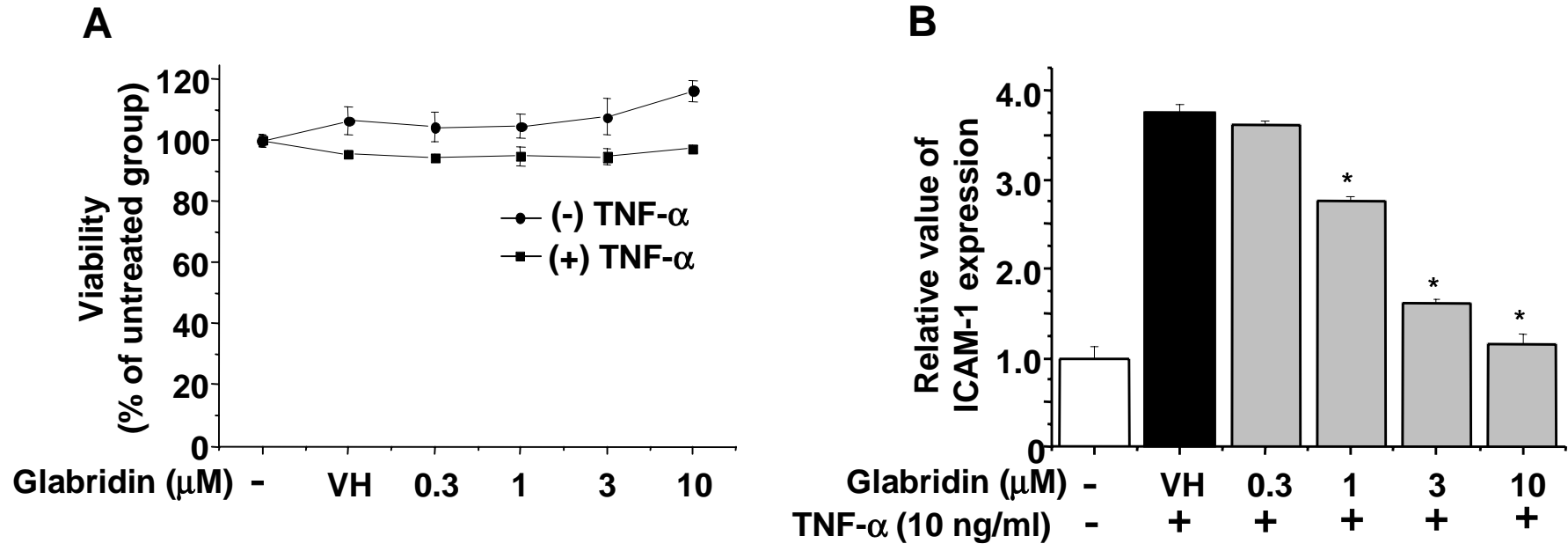


Fig. 3.

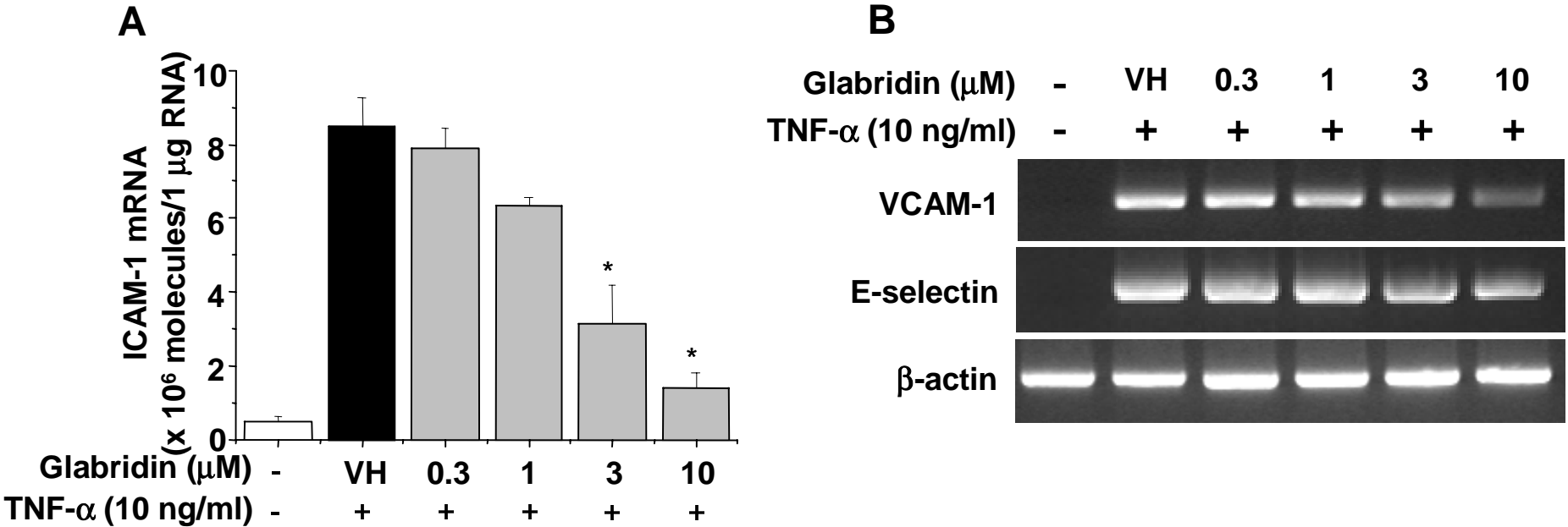
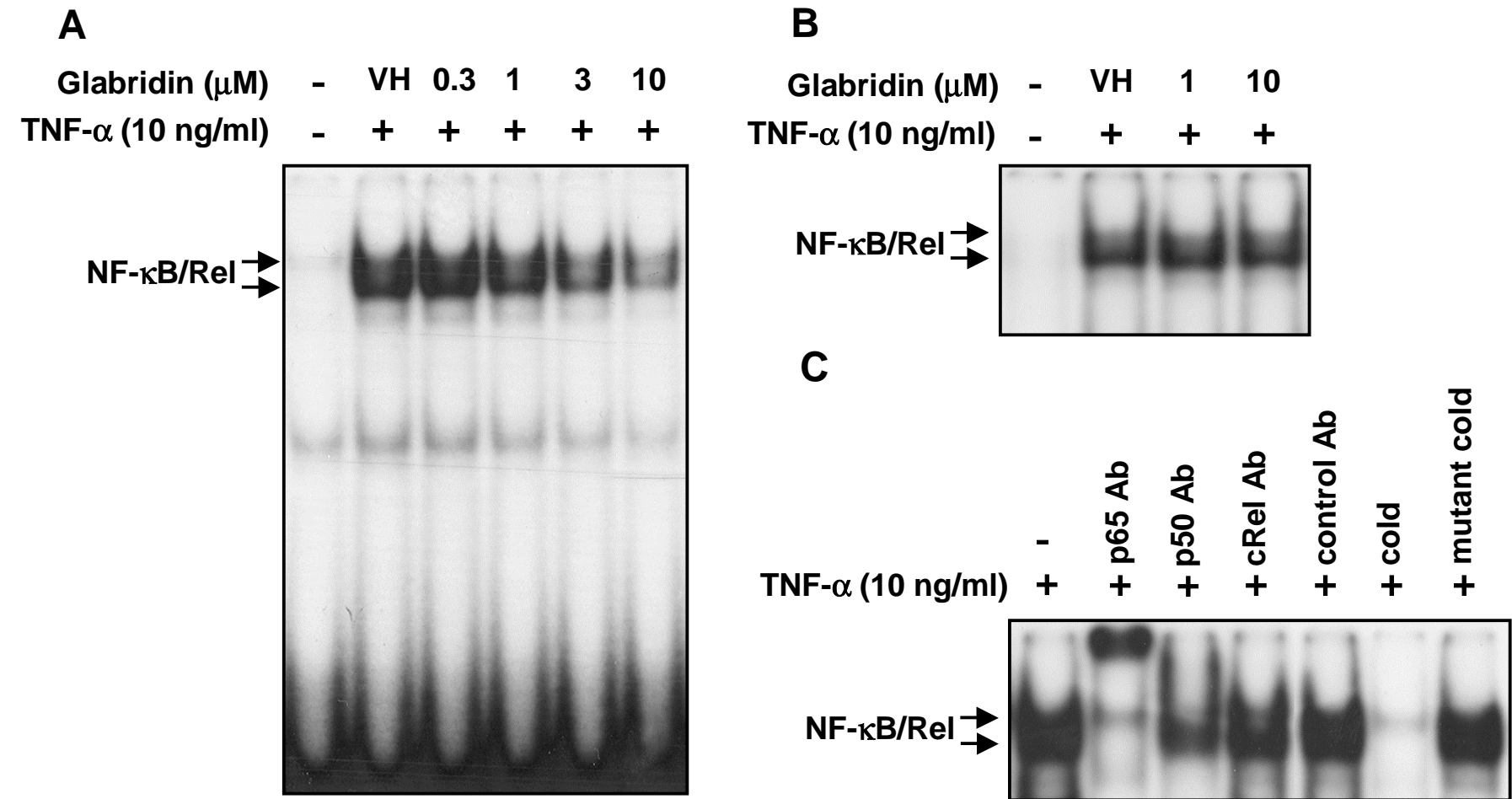


Fig. 4.



(continued)

Fig. 4.

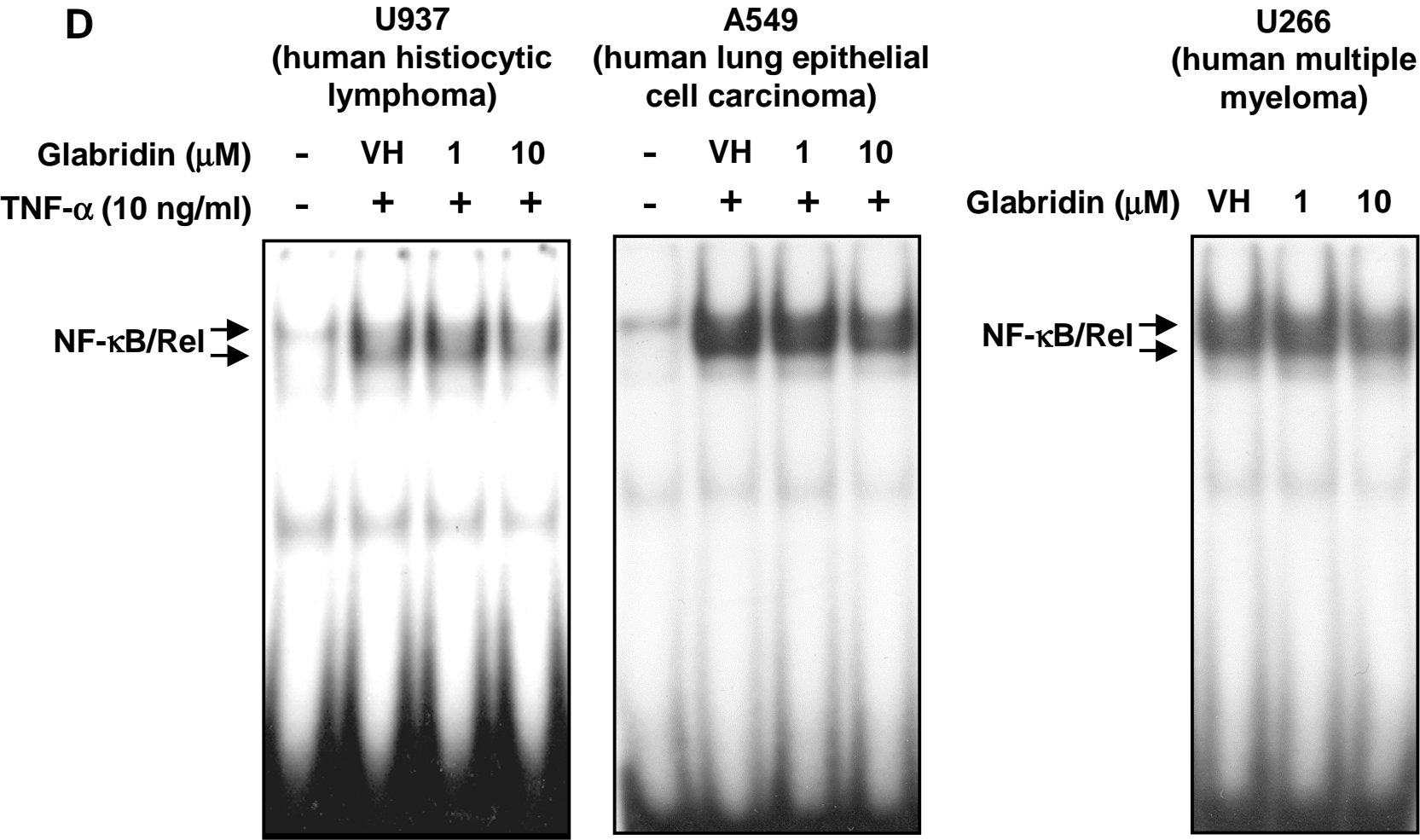


Fig. 5.

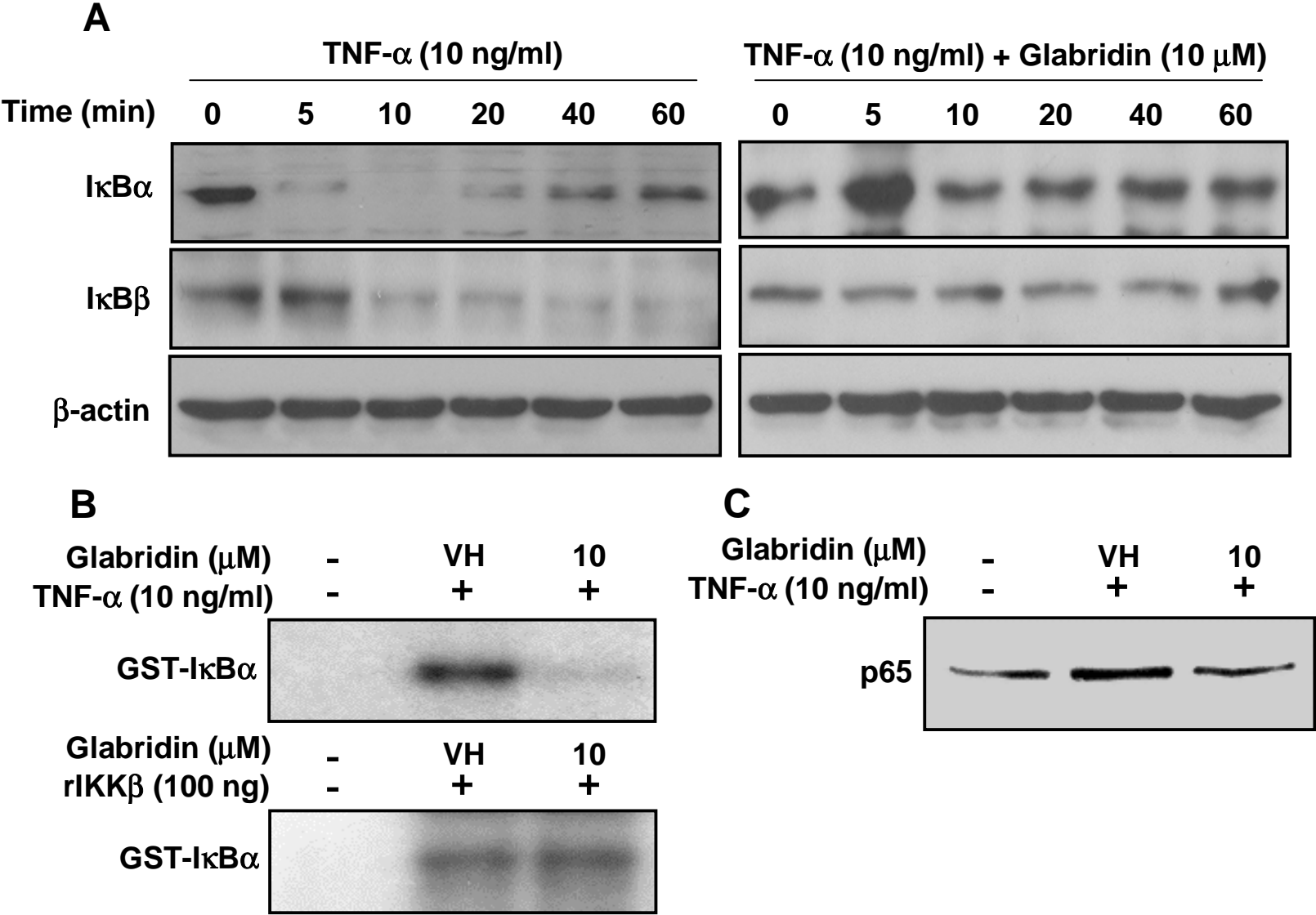


Fig. 6.

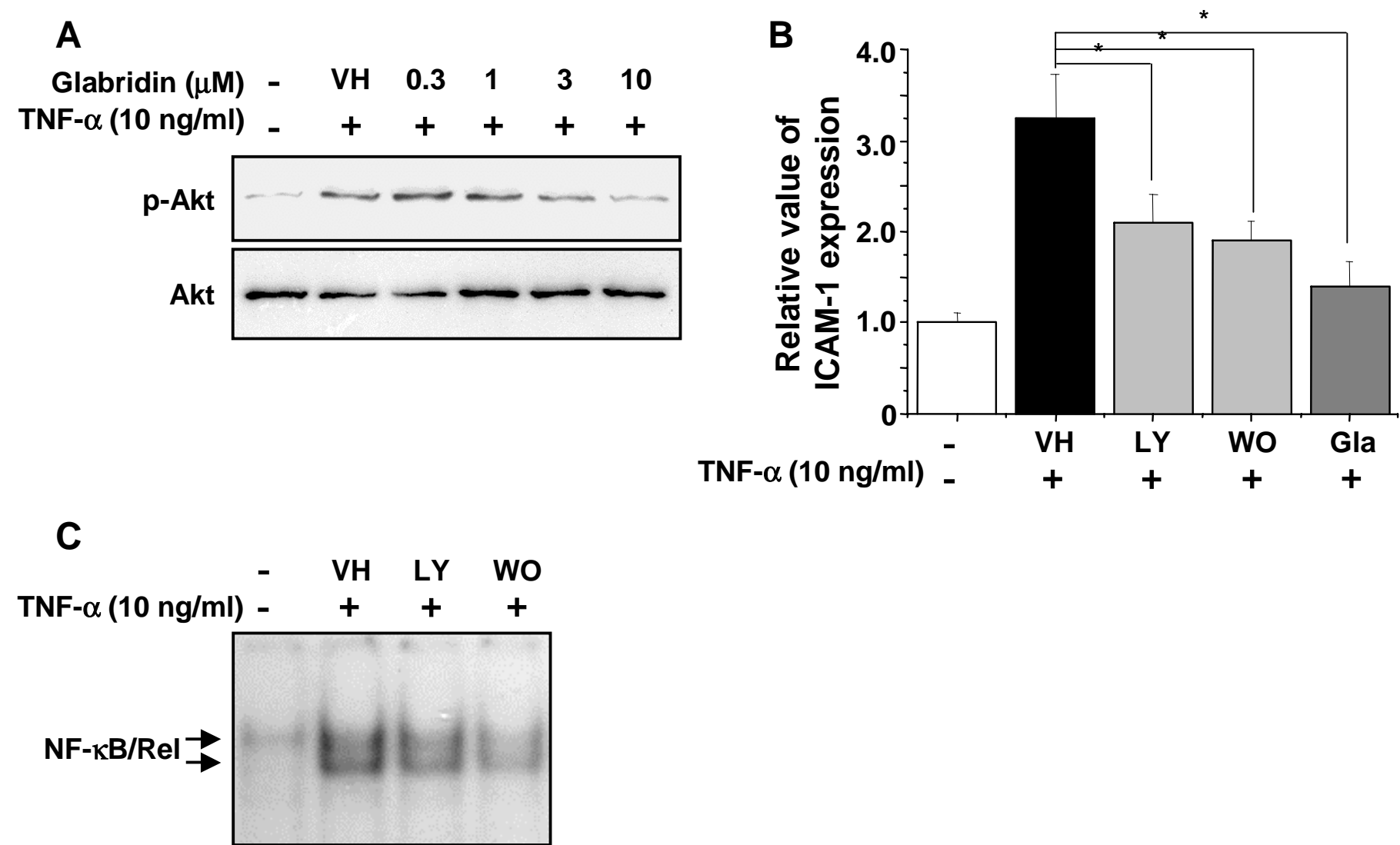


Fig. 7.

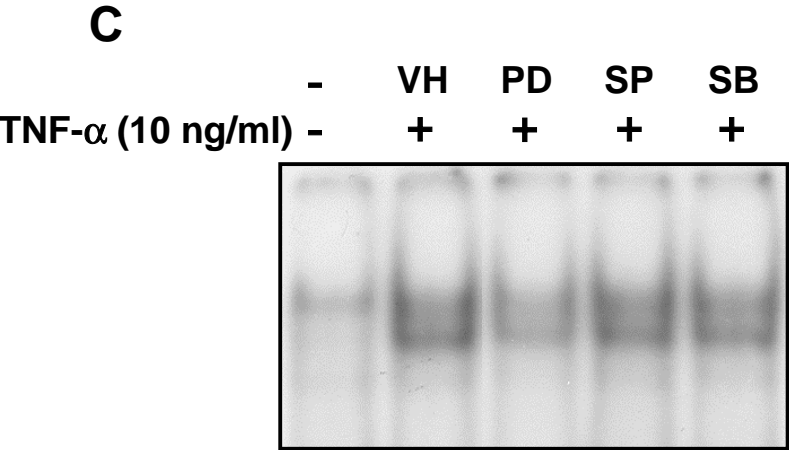
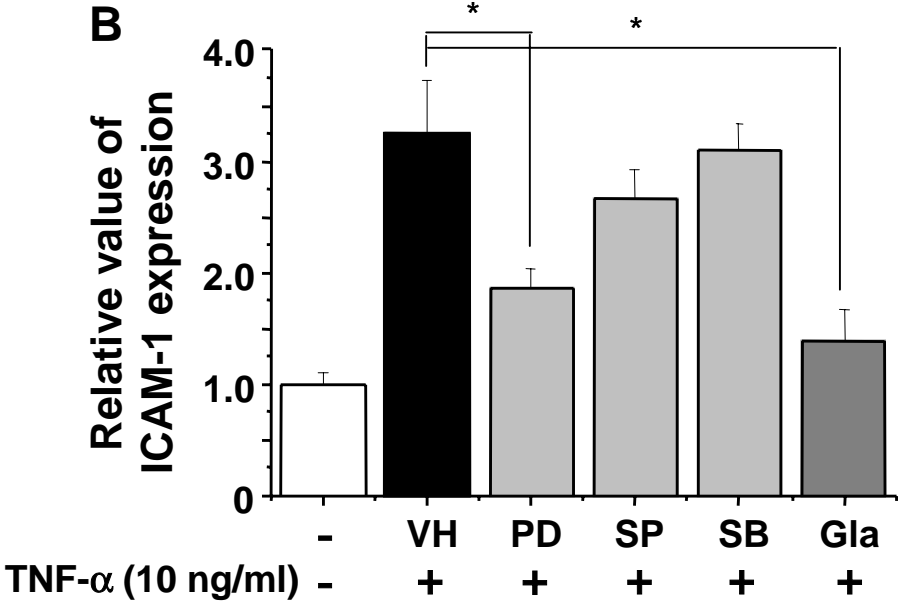
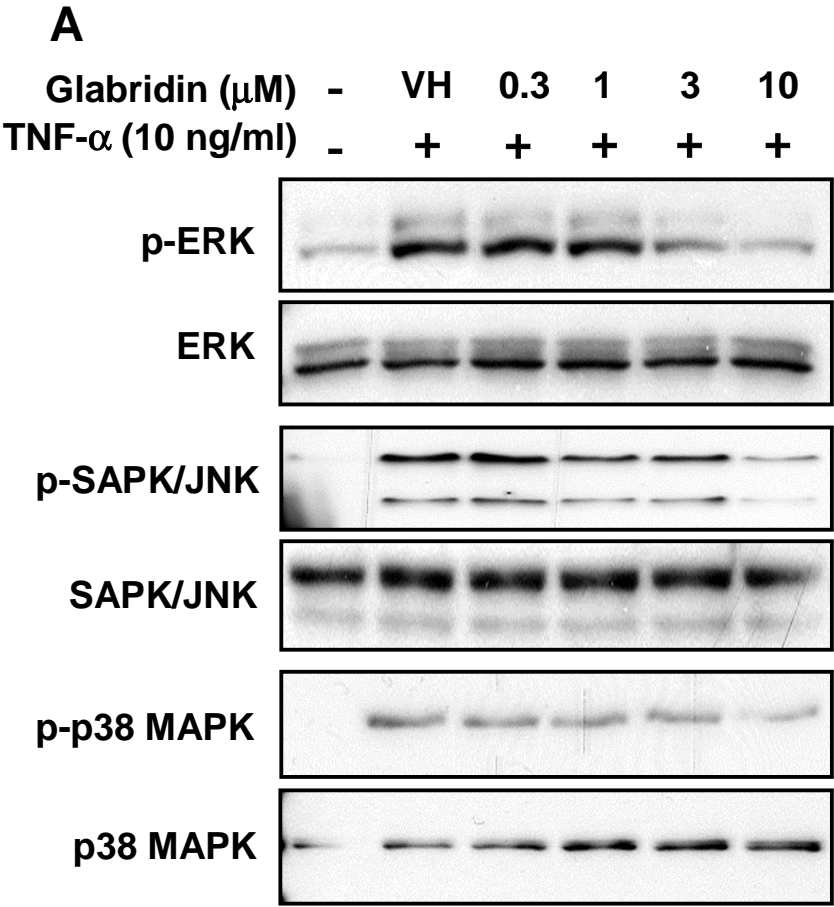


Fig. 8.

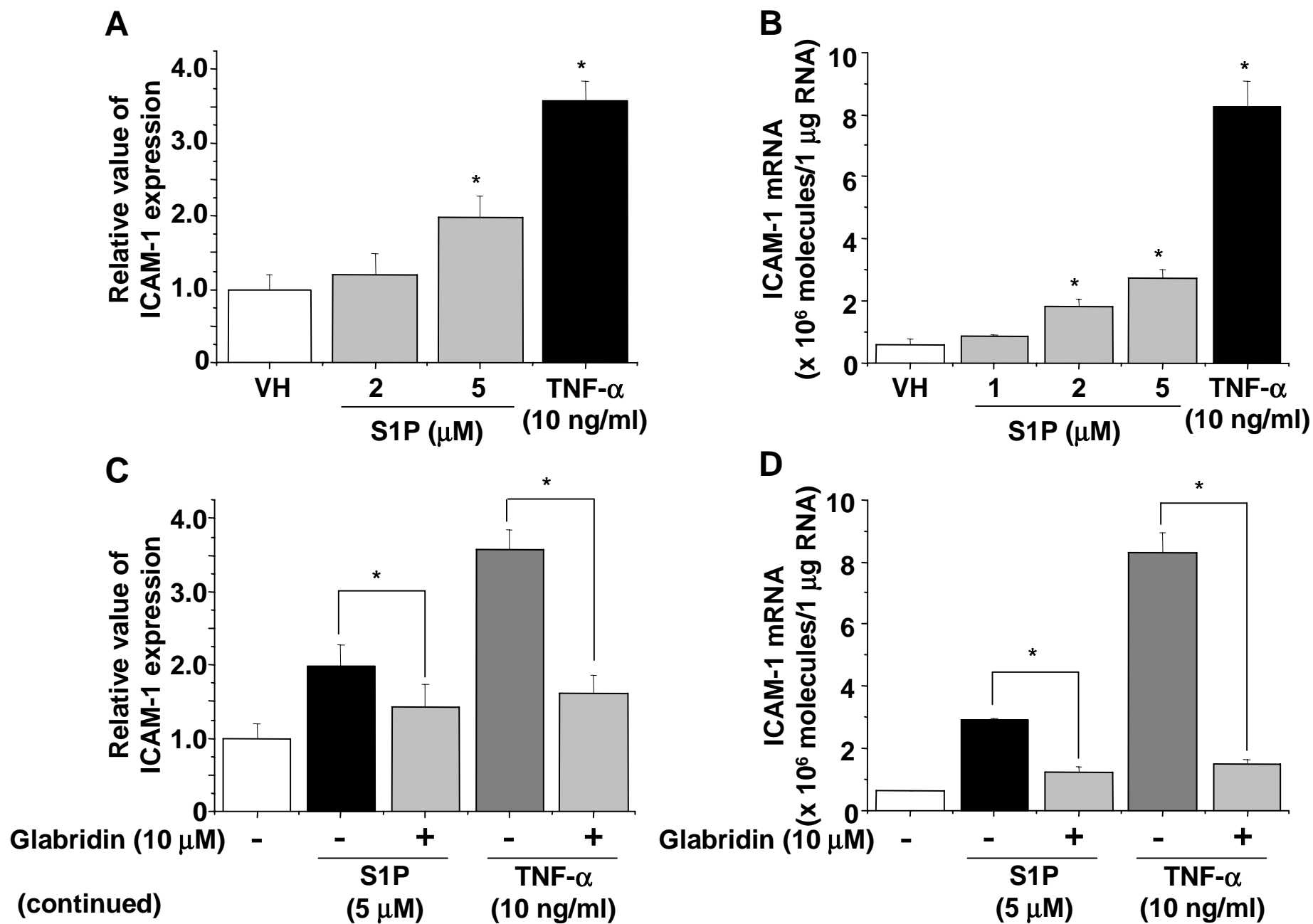


Fig. 8.

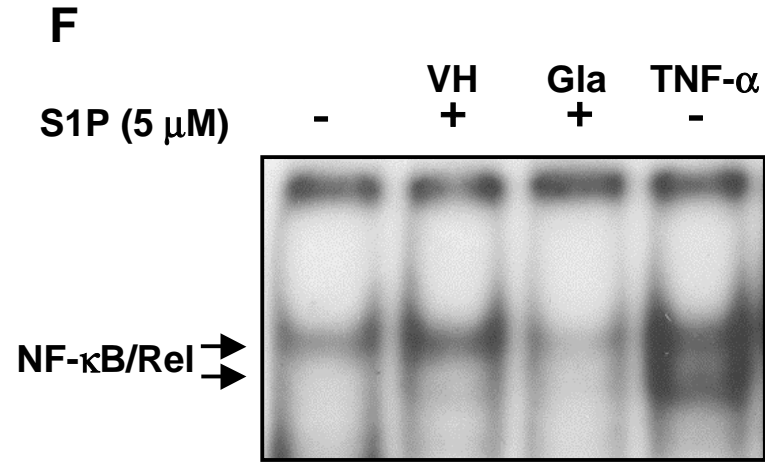
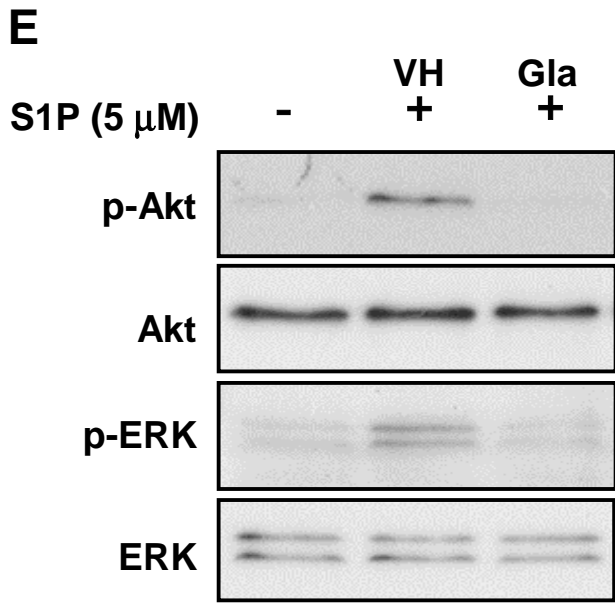


Fig. 9.

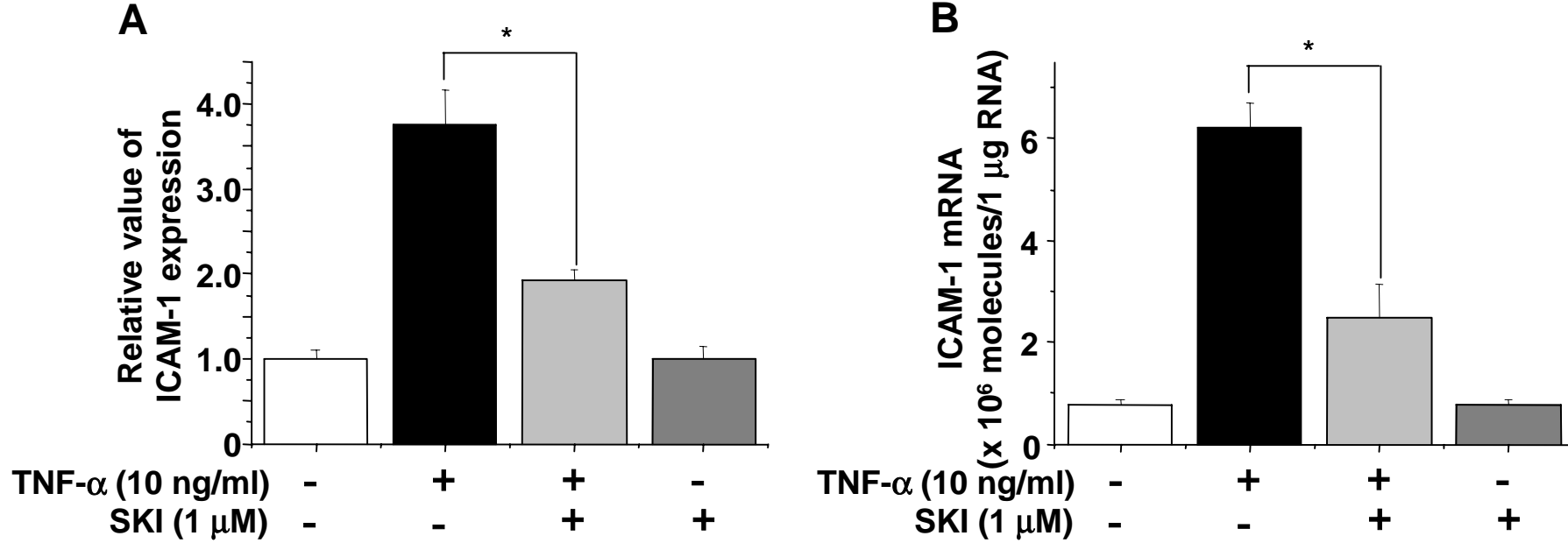


Fig. 10.

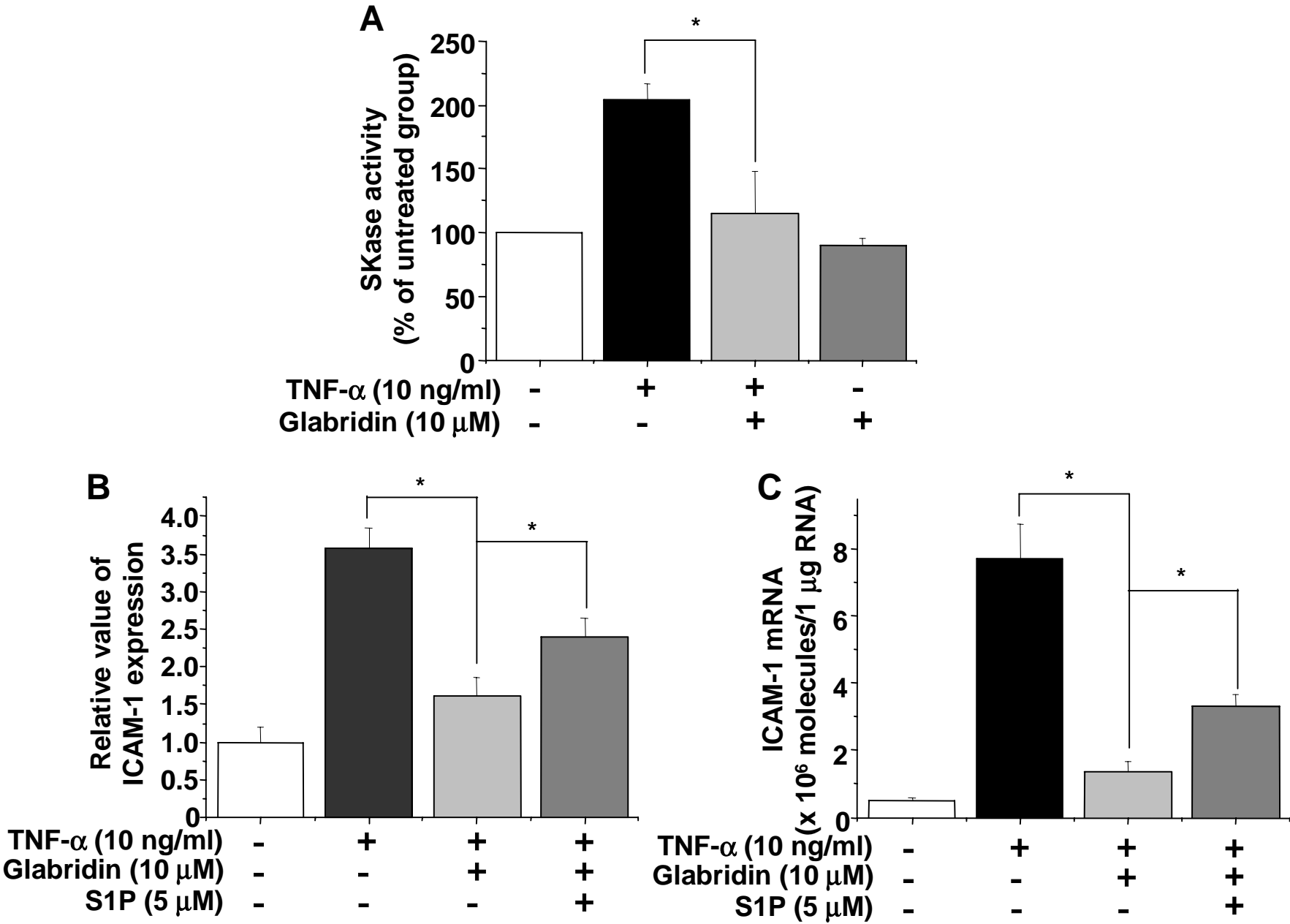


Fig. 11.

