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Glabridin, an Isoflavan from Licorice Root, Inhibits Inducible Nitric-Oxide Synthase Expression and Improves Survival of Mice in Experimental Model of Septic Shock

Jong Soon Kang, Yeo Dae Yoon, Ig Jun Cho, Mi Hwa Han, Chang Woo Lee, Song-Kyu Park, and Hwan Mook Kim

Bioevaluation Center, Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea

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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), a flavonoid present in licorice extract, is known to have antimicrobial, anti-inflammatory, and cardiovascular protective activities. In the present study, we report the inhibitory effect of glabridin on nitric oxide (NO) production and inducible nitric oxide (iNOS) gene expression in murine macrophages. Glabridin attenuated lipopolysaccharide (LPS)-induced NO production in isolated mouse peritoneal macrophages and RAW 264.7 cells, a mouse macrophage-like cell line. Moreover, iNOS mRNA expression was also blocked by glabridin treatment in LPS-stimulated RAW 264.7 cells. Further study demonstrated that the LPS-induced nuclear factor (NF)-κB/Rel DNA binding activity and NF-κB/Rel-dependent reporter gene activity were significantly inhibited by glabridin in RAW 264.7 cells and that this effect was mediated through the inhibition of inhibitory factor-κB degradation and p65 nuclear translocation. Moreover, reactive oxygen species generation was also suppressed by glabridin treatment in RAW 264.7 cells. In contrast, the activity of mitogen-activated protein kinases was unaffected by glabridin treatment. In animal model, in vivo administration of glabridin increased the rate of survival of LPS-treated mice and inhibited LPS-induced increase in plasma concentrations of nitrite/nitrate and tumor necrosis factor-α. Collectively, these data suggest that glabridin inhibits NO production and iNOS gene expression by blocking NF-κB/Rel activation and that this effect was mediated, at least in part, by inhibiting reactive oxygen species generation. Furthermore, in vivo anti-inflammatory effect of glabridin suggests a possible therapeutic application of this agent in inflammatory diseases.

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). Licorice contains glycyrrhizin, oleane triterpenoids, glucose, and flavonoids (Zhou et al., 2004). 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 is well known for its beneficial effects on the skin through an inhibitory effect on melanogenesis and inflammation (Yokota et al., 1998). It also has a wide range of biological activities, including antimicrobial, antiatherosclerotic, antinephritic, and cardiovascular protective activities (Fuhrman et al., 1997; Fukai et al., 2003; Zhou et al., 2004). Recently, it was reported that glabridin has a protective effect on low-density lipoprotein oxidation, and this contributes to the antiatherosclerotic effect of glabridin (Vaya et al., 1997; Belinky et al., 1998). Licorice extract has been known to have an anti-inflammatory effect, and various components of licorice extract, including glabridin, are reported to be involved in this effect by exerting an inhibitory effect on cyclooxygenase and lipoxygenase activities (Inoue et al., 1986; Yokota et al., 1998; Zhou et al., 2004).

Sepsis is a systemic response to infection, and septic shock is one of the most common causes of death in intensive care units (Titheradge, 1999). The most common cause of sepsis is an exposure to the structural component of a gram-negative bacterial membrane lipopolysaccharide (LPS), and key symp-

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ABBREVIATIONS: LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; NO, nitric oxide; PGE_2, prostaglandin E_2; NOS, nitric-oxide synthase; iNOS, inducible NOS; NF-κB/Rel, nuclear factor-κB/Rel; IκB, inhibitory factor-κB; RT-PCR, reverse transcription-polymerase chain reaction; AP-1, activator protein-1; CAT, chloramphenicol acetyltransferase; SAPK, stress-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; ROS, reactive oxygen species; NOx, nitrite/nitrate; MAP, mitogen-activated protein; SP600125, anthra[1,9-*cd*]pyrazol-6(2*H*)-one; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; PD98059, 2′-amino-3′-methoxyflavone; IKK, IκB kinase.

toms include hypotension and vasoplegia, which may lead to multiple organ dysfunction and ultimately death (Thiemermann, 1997). Bacterial LPS in the bloodstream induces the overexpression of various inflammatory mediators, such as interleukin-1β, tumor necrosis factor-α (TNF-α), nitric oxide (NO), and prostaglandin E_2 (PGE_2), and a large amount of inflammatory mediators produced in the body are thought to contribute to the LPS-induced symptoms of septic shock and mortality (Ando et al., 2000). Among these inflammatory mediators, NO is known to be closely associated with hypotension and hyporesponsiveness to vasoconstrictor stimuli in endotoxin-induced sepsis (Thiemermann, 1997; Titheradge, 1999). Moreover, mice lacking the inducible nitric-oxide synthase (iNOS) gene were reported to be resistant to the hypotension and death caused by LPS (MacMicking et al., 1995; Wei et al., 1995). Collectively, these reports indicate that NO plays a crucial role in endotoxin-induced septic shock and death.

NO is a short-lived free radical and intercellular messenger that mediates a variety of biological functions, including vascular homeostasis, neurotransmission, antimicrobial defense, and antitumor activities (Barbato and Tzeng, 2004). NO is known to be synthesized from L-arginine by nitric-oxide synthase (NOS). Three isoforms of NOS have been identified and are classified into two major categories, namely, constitutive and inducible NOS. Neuronal and endothelial NOS are expressed constitutively and are involved in regulating vascular tone (Achike and Kwan, 2003) and neurotransmission (Gibbs, 2003). They are activated by elevated levels of intracellular Ca^{2+}. On the other hand, iNOS, the high-output isoform, is functionally Ca^{2+}-independent and is expressed in a variety of cell types after transcriptional activation (Barbato and Tzeng, 2004). The iNOS gene expression is regulated mainly at the transcriptional level in macrophages, and the major transcriptional regulators of iNOS gene are the nuclear factor-κB (NF-κB)/Rel family of transcription factors that is also a key regulator of a variety of genes involved in immune and inflammatory response (Xie et al., 1994). In unstimulated cells, NF-κB/Rel exists in an inactive state in the cytoplasm complexed with the inhibitory protein called inhibitory factor-κB (IκB). Upon activation, IκB undergoes phosphorylation and degradation, and the NF-κB/Rel heterodimer is translocated into the nucleus where it binds to DNA and activates transcription (Shishodia and Aggarwal, 2004).

The objective of the present study was to assess the effect of glabridin on NO production and iNOS gene expression in macrophages. We also investigated the molecular mechanism responsible for the inhibitory effect of glabridin on iNOS gene expression. In addition, we examined the effect of glabridin on endotoxin-induced septic shock to assess its anti-inflammatory effect in vivo. In the present study, we demonstrated that glabridin inhibits NO production and iNOS gene expression by blocking NF-κB/Rel activation and protects mice against LPS-induced sepsis.

Materials and Methods

Chemicals, Animals, and Cell Culture. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Glabridin was purchased from Wako Pure Chemicals (Osaka, Japan), dissolved in dimethyl sulfoxide, and freshly diluted in culture media for all in vitro experiments. Chemical structure of glabridin was shown in Fig. 1. Virus-free female BDF1 mice were purchased from Dae Han Laboratory Animal Research Center Co., Ltd. (Chungbuk, Korea) and cared for as described previously (Kang et al., 2002). The mice used in this experiment were 6 to 7 weeks of age. For in vivo administration, glabridin was first dissolved in ethanol and diluted in 0.5% Tween 80 (v/v). Peritoneal macrophages were harvested by sterile peritoneal lavage using phosphate-buffered saline, washed, resuspended in culture medium, and plated. Nonadherent cells were removed by repeated washing after a 2-h incubation at 37°C. The peritoneal macrophages and RAW 264.7 cells (ATCC TIB71) were grown in RPMI 1640 and Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA), respectively, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in 5% CO_2 humidified air.

Nitrite Quantification. Mouse peritoneal macrophages and RAW 264.7 cells were plated at 5×10^6 and 5×10^5 cells/ml, respectively and stimulated with LPS (200 ng/ml from *Salmonella typhosa*) in the presence and absence of glabridin (0.3, 1, 3, or 10 μM) for 24 h. The isolated supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. $NaNO_2$ was used to generate a standard curve, and nitrite production was determined by measuring optical density at 540 nm.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). The expressions of the mRNA transcripts of iNOS (forward primer: 5′-CTGCAGCACTTGGATCAGGAACCTG-3′, reverse primer: 5′-GGGAGTAGCCTGTGTGCACCTGGAA-3′), and β-actin (forward primer: 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, reverse primer: 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′) were evaluated by RT-PCR as described previously (Jeon et al., 2000) with slight modifications. Briefly, total RNA was isolated using Tri reagent (Molecular Research Center, Cincinnati, OH) as described previously (Chomczynski and Mackey, 1995). Equal amounts of RNA were reverse-transcribed into cDNA using oligo$(dT)_{15}$ primers. Samples were heated to 94°C for 5 min and cycled 30 times at 94°C for 30 s, 56°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. PCR products were electrophoresed in 3% NuSieve 3:1 gels (FMC Bioproducts, Rockland, ME) and followed by ethidium bromide staining and photography. Band intensities were quantified using ImageQuant software (Amersham Biosciences Inc., Piscataway, NJ).

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared as described previously (Jeon et al., 2000). The protein content of the nuclear extracts was determined using a Bio-Rad protein assay kit according to the manufacturer's instruction (Bio-Rad, Hercules, CA). The oligonucleotide sequence for NF-κB/Rel, AP-1, and octamer was as follows: 5′-GATCTCAGAGGGGACTTTC-CGAGAGA-3′, 5′-GATCTGCATGAGTCAGACACACA-3′, and 5′-GATCTTCTAGAGGATCATGCAAATGATCA-3′ (Kang et al., 2002). Double-stranded oligonucleotides were end-labeled with [γ-^{32}P]ATP. Nuclear extracts (5 μg) were incubated with 2 μg 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.

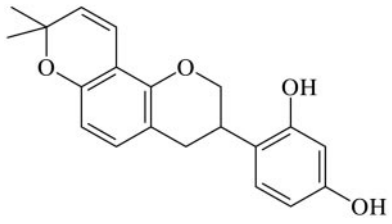

Fig. 1. Chemical structure of glabridin.

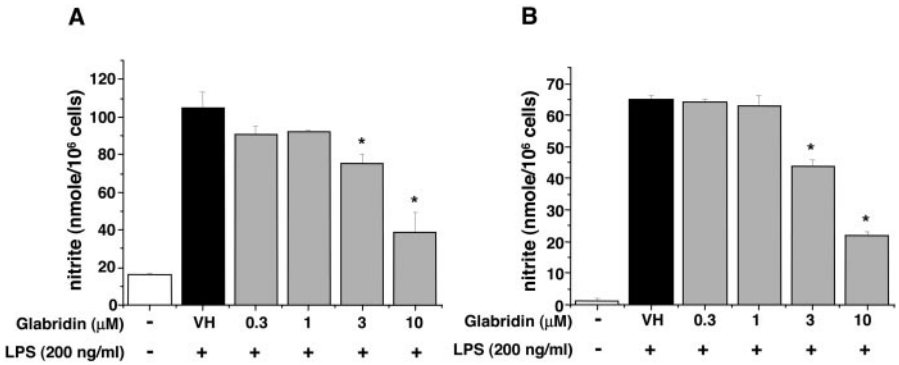

Fig. 2. Inhibition of nitrite production by glabridin in LPS-stimulated peritoneal macrophages and RAW 264.7 cells. Peritoneal adherent cells (A) and RAW 264.7 cells (B) were pretreated with the indicated concentrations of glabridin for 1 h before being incubated with LPS (200 ng/ml) for 24 h. The culture supernatants were subsequently isolated and analyzed for nitrite production. Each column shows the mean ± S.D. of quadruplicate determinations. Significance was determined using Student's t test versus the control group (*, $p < 0.01$).

Transient Transfection and Chloramphenicol Acetyltransferase (CAT) Reporter Gene Assay. p(NF-κB)$_3$CAT plasmid has been described previously (Kang et al., 2002). Transient transfection was performed using LipofectAMINE Plus reagent (Invitrogen). Eighteen hours after transfection, cells were treated with the indicated concentrations of glabridin (0.3, 1, 3, or 10 μM) 1 h before the treatment of LPS (200 ng/ml), harvested 24 h after LPS treatment, and lysed. The CAT enzyme expression levels were determined using a CAT enzyme-linked immunosorbent assay kit according to the manufacturer's instruction (Roche Applied Science, Mannheim, Germany).

Western Immunoblot Analysis. Twenty micrograms of whole cell lysate (for p-SAPK/JNK, SAPK/JNK, p-p38, and p38), cytosolic extract [for IκBα, IκBβ, and IκB kinase (IKK)α], and nuclear extract (for p65) were separated by 10% SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membrane (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). The membranes were preincubated for 1 h at room temperature in Tris-buffered saline, pH 7.6, containing 0.05% Tween 20 and 5% nonfat milk. The nitrocellulose membranes were then incubated with specific antibodies against p-SAPK/JNK, SAPK/JNK, p-p38, p38, IKKα, p65 (Cell Signaling Technology Inc., Beverly, MA), IκBα, or IκBβ (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Immunoreactive bands were then detected by incubating with conjugates of anti-rabbit IgG with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham Biosciences UK, Ltd.).

Determination of Reactive Oxygen Species (ROS). The production of ROS was determined using 2′7′-dichlorofluorescin diacetate, an oxidant-sensitive fluorescent probe, and by flow cytometry as described previously (Kang et al., 2002). Briefly, cells were preincubated with Hank's balanced salt solution in the presence of glabridin (1 and 10 μM) or ascorbic acid (50 μM) for 30 min at 37°C in a water bath. After being incubated for 15 min with 20 mM 2′7′-dichlorofluorescin diacetate, 1 mM H_2O_2 was treated, and incubation continued for an additional 15 min. The relative green dichlorofluorescin fluorescence within the living cells was measured by flow cytometry (Bio-Rad).

Survival Study. The female BDF1 mice were divided into four groups, and glabridin and vehicle [5% Tween 80 (v/v) and 2% ethanol] was administrated intraperitoneally at 1 and 10 mg/kg 24 and 2 h before LPS administration. Glabridin was administrated twice to increase availability and efficacy of drug. LPS (from *S. typhosa*) was administrated intraperitoneally at a dose of 15 mg/kg, and survival of mice was monitored every day for 4 days.

Measurement of Plasma Concentration of Nitrite/Nitrate (NOx) and TNF-α. For measurement of plasma NOx and TNF-α concentrations, whole blood samples were withdrawn from mice by orbital puncture, and plasmas were prepared. For measurement of plasma NOx concentration, plasma samples were ultrafiltered through 10,000 molecular weight cutoff filter (Microcon YM-10; Millipore Corporation, Bedford, MA) to eliminate proteins, and plasma NOx concentrations were determined using Nitric Oxide (NO_2^-/NO_3^-) assay kit (R&D Systems, Minneapolis, MN) according to manufacturer's instruction. Plasma TNF-α concentration was measured by enzyme-linked immunosorbent assay using a protocol supplied by R&D Systems.

Statistical Analysis. The mean ± S.D. was determined for each treatment group in each experiment. Data were analyzed by analysis of variance, and Student's t test was used for comparisons of multiple comparisons. The criterion for statistical significance was set at $p < 0.05$ or $p < 0.01$.

Results

Effect of Glabridin on NO Production in Mouse Macrophages. To investigate the effect of glabridin on NO production, we measured the accumulation of nitrite, the stable metabolite of NO, in the culture media using Griess reagent. Accumulation of nitrite in media can be detected about 16 to 20 h after LPS treatment in mouse peritoneal macrophages and RAW 264.7 cells using Griess reagent, and the rate of accumulation is maintained at a high level until about 24 to 30 h. In this study, we measured nitrite accumulation 24 h

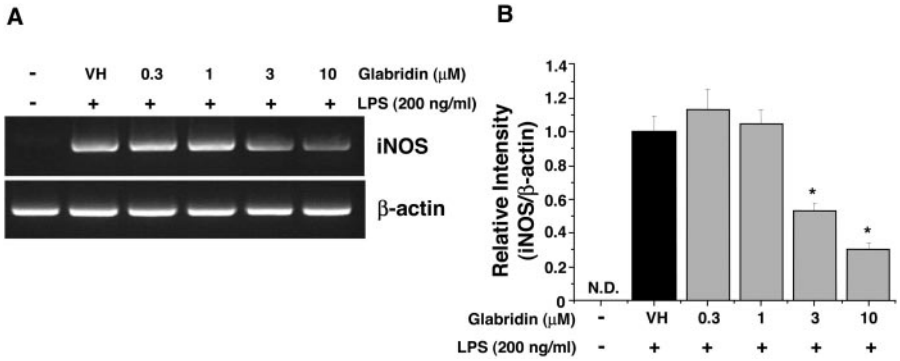

Fig. 3. Inhibition of iNOS mRNA expression by glabridin in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with the indicated concentration of glabridin for 1 h before being incubated with LPS (200 ng/ml) for 6 h. Total RNA was isolated, and iNOS mRNA expression was determined by competitive RT-PCR. PCR products were electrophoresed in 3% NuSieve 3:1 gels and stained with ethidium bromide (A). The ratio of band intensities of iNOS to β-actin was calculated and graphed (B). Significance was determined using Student's t test versus the control group (*, $p < 0.01$). N.D., not detected.

after LPS treatment to assess the blocking effect of glabridin on NO production in LPS-stimulated isolated peritoneal macrophages and RAW 264.7 cells. Isolated peritoneal macrophages and RAW 264.7 cells were treated with the indicated concentrations of glabridin (0.3, 1, 3, or 10 μM) for 1 h before being incubated with LPS (200 ng/ml). As shown in Fig. 2A, LPS (200 ng/ml) alone increases nitrite generation to 6.6 times the basal level in isolated mouse peritoneal macrophages, and this induction was concentration-dependently suppressed by glabridin (34% inhibition at 3 μM and 74% inhibition at 10 μM). In RAW 264.7 cells, LPS (200 ng/ml) caused marked increase of nitrite generation versus the untreated control, and this induction was also inhibited by glabridin treatment in a dose-dependent manner (33% inhibition at 3 μM and 67% inhibition at 10 μM) (Fig. 2B). The concentration and duration of glabridin treatment used in these studies had no significant effect on the viability of isolated peritoneal macrophages and RAW 264.7 cells (data not shown).

Effect of Glabridin on iNOS mRNA Expression in LPS-Stimulated RAW 264.7 Cells. To investigate whether the inhibitory effect of glabridin on NO production is due to the reduced expression of iNOS gene, we assessed the effect of glabridin on iNOS mRNA expression by RT-PCR. The level of iNOS mRNA expression reaches maximum level at about 6 h after LPS treatment. Therefore, we measured iNOS mRNA level 6 h after LPS treatment to examine the blocking effect of glabridin on LPS-induced iNOS mRNA expression in RAW 264.7 cells. The expression of iNOS mRNA was hardly detectable in unstimulated cells; however, RAW 264.7 cells expressed a high level of iNOS mRNA when stimulated with LPS (200 ng/ml) for 6 h. Furthermore, glabridin inhibited LPS-induced expression of iNOS mRNA in a dose-dependent manner (47% inhibition at 3 μM and 70% inhibition at 10 μM) (Fig. 3), suggesting that the inhibitory effect of glabridin on NO production was mediated, at least in part, by the inhibition of gene expression of iNOS. In contrast to iNOS, the level of β-actin mRNA expression remained the same under these conditions.

Effect of Glabridin on LPS-Induced NF-κB/Rel DNA Binding and Transcriptional Activity in RAW 264.7 Cells. NF-κB/Rel is an important transcriptional regulator of iNOS and plays a crucial role in the immune and inflammatory responses. To investigate the intracellular mechanism responsible for the inhibitory effect of glabridin on iNOS gene expression, we examined the effect of glabridin on NF-κB/Rel activity using electrophoretic mobility shift assay and CAT reporter gene assay. The DNA binding activity of NF-κB/Rel reaches maximum level at about 1 h after LPS treatment in RAW 264.7 cells, and therefore, we measured the effect of glabridin on LPS-induced NF-κB/Rel DNA binding activity at this time point. Treatment of LPS (200 ng/ml) caused a significant increase in the DNA binding activity of NF-κB/Rel within 1 h (Fig. 4A). In the presence of glabridin, LPS-induced NF-κB/Rel DNA binding was markedly suppressed in a concentration-dependent manner (Fig. 4A). Because AP-1 and octamer are also involved in the expression of iNOS gene, we examined the effect of glabridin on DNA binding activity of AP-1 and octamer. Although AP-1 binding was substantially up-regulated by LPS (200 ng/ml), this AP-1 binding was not inhibited by glabridin at low concentrations and was only slightly inhibited at highest concentration (10 μM) (Fig. 4B). Octamer binding was unaffected by either LPS or glabridin treatment (Fig. 4B). To further confirm the inhibitory effect of glabridin on NF-κB/Rel, we examined the effect of glabridin on the NF-κB/Rel-dependent reporter gene expression. We performed reporter gene assay using p(NF-κB)$_3$CAT plasmid, which was generated as described previously (Kang et al., 2002). RAW 264.7 cells transfected with p(NF-κB)$_3$CAT plasmid were stimulated with LPS (200 ng/ml) in the presence or absence of glabridin. CAT enzyme expression was markedly increased 24 h after LPS treatment, and glabridin significantly reduced the LPS-induced increase in NF-κB/Rel-dependent CAT enzyme expression in a concentration-dependent manner (Fig. 5).

Effect of Glabridin on Degradation of IκBα and IκBβ and Nuclear Translocation of p65 Subunit of NF-κB/Rel in LPS-Stimulated RAW 264.7 Cells. The activation of NF-κB/Rel occurs via nuclear translocation of Rel family proteins and this is preceded by the phosphorylation and degradation of IκBs by IκB kinases (Stancovski and Baltimore, 1997). Therefore, to further investigate the mechanism responsible for the inhibitory effect of glabridin on NF-κB/Rel activity, we examined the effect of glabridin on IκB degrada-

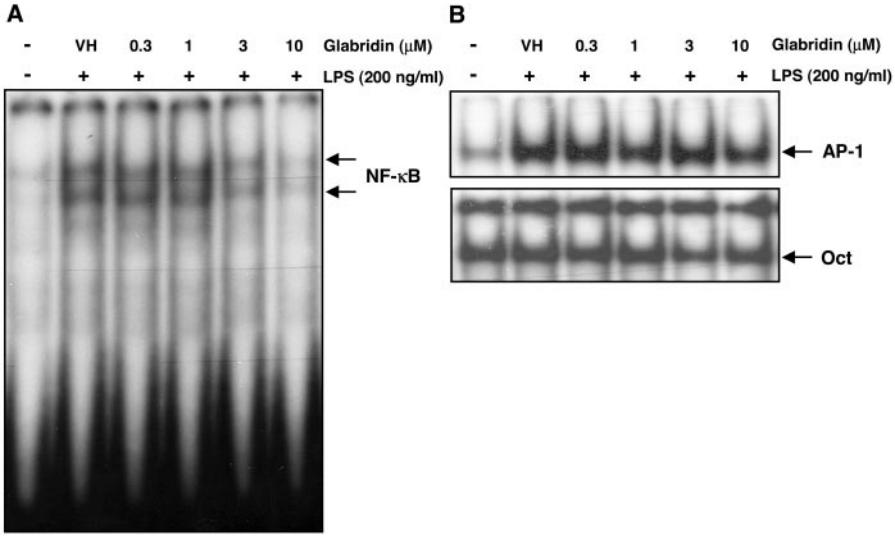

Fig. 4. Effect of glabridin on the binding of transcription factors in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with the indicated concentrations of glabridin for 1 h before being incubated with LPS (200 ng/ml) for 1 h. Nuclear extracts were then prepared, and NF-κB/Rel (A) and AP-1 and octamer (B) binding was determined by electrophoretic mobility shift assay.

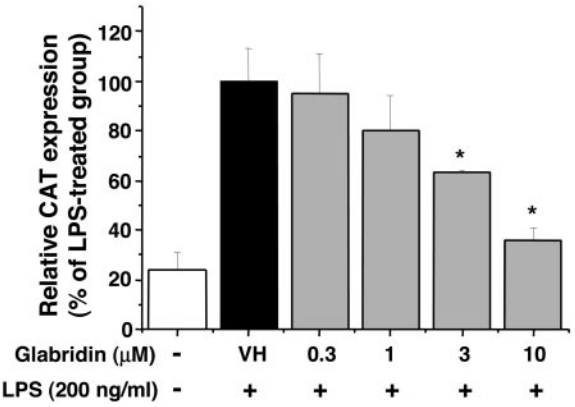

Fig. 5. Inhibition of NF-κB/Rel-dependent reporter gene expression by glabridin in LPS-stimulated RAW 264.7 cells. Cells were transiently transfected with $p(NF\text{-}\kappa B)_3CAT$ containing three copies of the NF-κB/Rel binding site, treated with the indicated concentrations of glabridin and LPS (200 ng/ml) for 24 h, and assayed for CAT expression using a CAT enzyme-linked immunosorbent assay kit. Each column shows the mean ± S.D. of quadruplicate determinations. Significance was determined using Student's *t* test versus the control group (*, $p < 0.01$).

tion and p65 nuclear translocation using Western immunoblot analysis. As mentioned above because degradation of IκBs and nuclear translocation of p65 are followed by NF-κB/Rel activation, we detected the effect of glabridin on IκB degradation and p65 nuclear translocation at earlier time points than that of NF-κB/Rel DNA binding assay. The degradation of IκBα was detected 30 min after LPS (200 ng/ml) treatment, and the level of these proteins was recovered by glabridin treatment (Fig. 6A). As shown in Fig. 6A, IκBβ was also degraded by LPS, and the pretreatment of cells with glabridin suppressed the LPS-induced IκBβ degradation. However, the level of IKKα was unaffected by either LPS or glabridin treatment (Fig. 6A). Next, we examined the effect of glabridin on nuclear translocation of the p65 subunit of NF-κB/Rel. Figure 6B shows that the nuclear translocation of the p65 subunit of NF-κB/Rel was inhibited by glabridin treatment in LPS-stimulated RAW 264.7 cells.

Effect of Glabridin on the LPS-Induced Activation of SAPK/JNK and p38 MAP Kinase in RAW 264.7 Cells. SAPK/JNK and p38 MAP kinase are known to be important for the expression of iNOS gene (Chan and Riches, 1998; Guan et al., 1999; Jeon et al., 2000). We also confirmed the importance of SAPK/JNK and p38 MAP kinase on NO production in RAW 264.7 cells using specific inhibitors of each kinase, SP600125 (10 μM) and SB203580 (30 μM), respectively. As shown in Fig. 7B, SP600125 and SB203580 suppressed nitrite generation in LPS-stimulated RAW 264.7 cells, whereas PD98059 (30 μM), a specific inhibitor of mitogen-activated protein kinase kinase-1, had no effect on nitrite generation. Therefore, we examined the effect of glabridin on LPS-induced activation of SAPK/JNK and p38 MAP kinase. Since activation of MAP kinase by LPS reaches a maximum level between 15 and 30 min after LPS treatment in RAW 264.7 cells, we performed these assays at 30 min after LPS treatment. Figure 7A shows that glabridin had no effect on the activation of SAPK/JNK and p38 MAP kinase in RAW 264.7 cells.

Effect of Glabridin on Reactive Oxygen Species Production in RAW 264.7 Cells. ROS are known to be involved in the activation of NF-κB/Rel (Flohe et al., 1997). To investigate the mechanism responsible for the inhibitory effect of glabridin on NF-κB/Rel activation, we examined the effect of glabridin on H_2O_2-induced ROS production. As shown in Fig. 8, 1 mM H_2O_2 markedly increased the production of ROS in RAW 264.7 cells, and this was completely blocked by glabridin treatment. Ascorbic acid (50 μM), a potent antioxidant, also blocked ROS production in H_2O_2-treated RAW 264.7 cells.

Effect of in Vivo Exposure of Glabridin on LPS-Treated Mice. To assess the in vivo anti-inflammatory effect of glabridin, we examined the effect of glabridin on mortality in mouse model of sepsis. BDF1 mice have been used in a variety of sepsis models, such as LPS-induced sepsis model (Hahn et al., 1998; Nose et al., 1998), burn sepsis model and cecal ligation (Gamelli et al., 1994; Miles et al., 1994), and puncture model (O'Reilly et al., 1992; Miles et al., 1994). To synchronize the animals used in all experiments, we used BDF1 mice in sepsis model. We used LPS isolated from *S. typhosa*, and the amount of LPS used in mouse model of sepsis was determined according to previous reports (Liu et al., 2003; Kang et al., 2004). As shown in Fig. 9, the administration of a high dose of LPS (15 mg/kg i.p.) to female BDF1 mice resulted in a survival rate of 43% after 2 days; however, the survival rate of mice was increased to 57% when treated with 1 mg/kg glabridin (i.p.) (Fig. 9). Moreover, treatment of a high dose of glabridin (10 mg/kg i.p.) nearly completely blocked LPS-induced mortality of mice (86%). We also assessed the effect of glabridin on plasma concentration of NOx and TNF-α in LPS-treated mice. As shown in Table 1, the concentration of NOx and TNF-α in plasma in vehicle-treated mice were 0.38 μM and 0.11 ng/ml, respectively ($n = 4$). After 8 h of LPS administration (200 μg/kg), the plasma levels of NOx and TNF-α were increased to 6.03 μM and 1.6 ng/ml, respectively. Consistent with previous results, treatment with glabridin attenuated LPS-induced increase in the

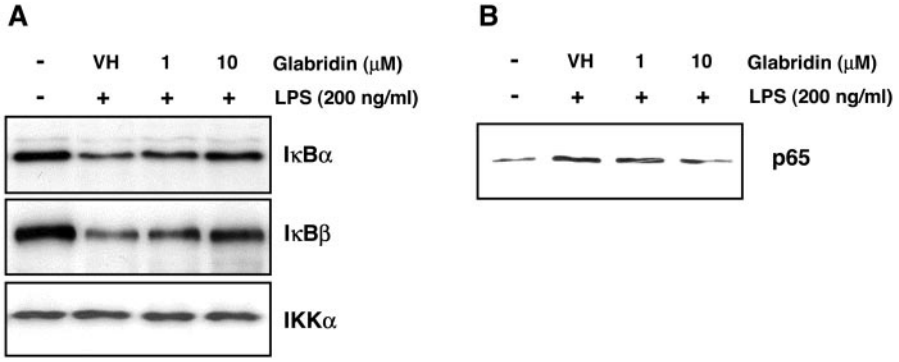

Fig. 6. Inhibition of degradation of IκBα and IκBβ and nuclear translocation of p65 subunit of NF-κB/Rel by glabridin in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with the indicated concentrations of glabridin for 1 h, incubated with LPS (200 ng/ml) for 30 min, and then assayed for the degradation of IκBs and nuclear translocation of p65 by Western immunoblot analysis as described under *Materials and Methods*.

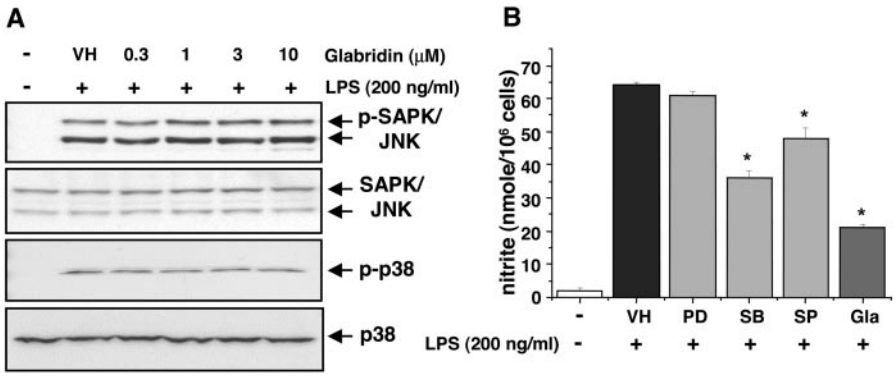

Fig. 7. Effect of glabridin on the LPS-induced activation of SAPK/JNK and p38 MAP kinase in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of glabridin for 1 h, incubated with LPS (200 ng/ml) for 30 min, and then assayed for activation of SAPK/JNK and p38 MAP kinase (A) using Western immunoblot analysis. Effect of PD98059 (PD, 30 μM), SB203580 (SB, 30 μM), or SP600125 (SP, 10 μM) on nitrite generation in LPS-stimulated RAW 264.7 cells (B). Each column shows the mean ± S.D. of quadruplicate determinations. Significance was determined using Student's t test versus the control group (*, $p < 0.01$).

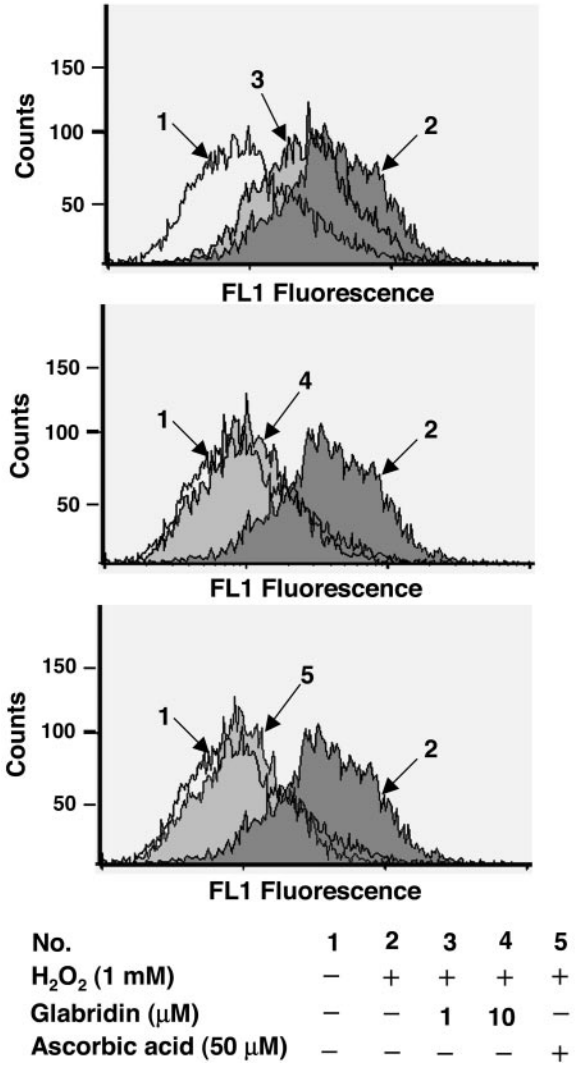

Fig. 8. Inhibition of ROS production by glabridin in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of glabridin or ascorbic acid for 30 min. After being incubated with 1 mM H_2O_2 for 15 min, the cells were analyzed using a flow cytometer. Fluorescence histograms are shown.

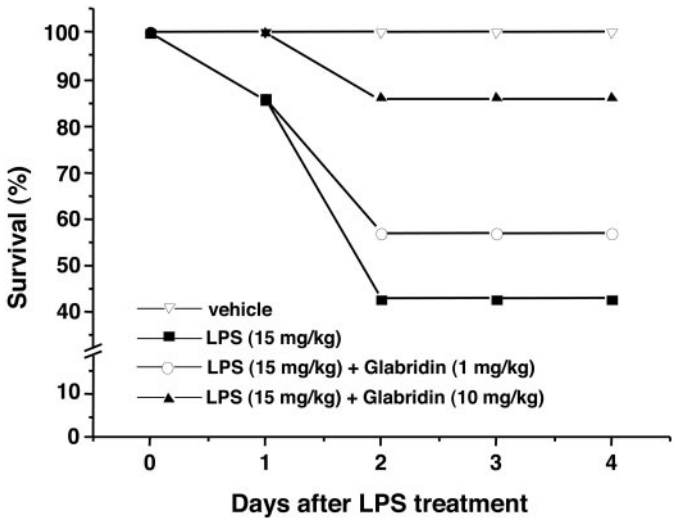

Fig. 9. Effect of glabridin on survival rate of mice treated with LPS (15 mg/kg). Different groups ($n = 7$) of animals were treated with vehicle (▽), vehicle plus LPS (15 mg/kg, i.p.) (■), or LPS (15 mg/kg i.p.) plus glabridin (1 or 10 mg/kg at 24 and 2 h before LPS treatment) (○ or ▲). The survival rate was measured everyday throughout this experiment.

plasma concentrations of NOx and TNF-α in a dose-dependent manner (Table. 1). The levels of NOx and TNF-α in plasma were decreased to 1.51 μM and 0.73 ng/ml, respectively, by the treatment with a high dose of glabridin (10 μM). In addition, we isolated peritoneal macrophages from these mice cultured 24 h and examined NO production by these cells according to the protocol used in our previous report (Kang et al., 2004). Peritoneal macrophages isolated from LPS (200 μg/kg)-treated mice produced large amounts of nitrite (31.868 nmol/10^6 cells) compared with untreated mice (1.134 nmol/10^6 cells) (Table. 1). However, Table 1 shows that treatment with 10 mg/ml glabridin substantially decreased NO production by isolated peritoneal macrophages (4.369 nmol/10^6 cells).

Discussion

Glabridin has been known to have a variety of beneficial effects including antimicrobial, anti-inflammatory, antiatherosclerotic, and antinephritic activities (Fuhrman et al., 1997; Yokota et al., 1998; Zhou et al., 2004). Until now, it has been known that the anti-inflammatory effect of glabridin was due to its inhibitory effect on cyclooxygenase activity (Yokota et al., 1998). In the present study, we clearly demonstrated that glabridin inhibits NO production and iNOS gene expression in LPS-stimulated macrophages. Because NO plays an important role in inflammation, the inhibitory effect of glabridin on iNOS gene expression suggests an additional possible mechanism responsible for the anti-inflammatory action of glabridin. A series of recent publications indicated that the overproduction of NO is implicated in the

TABLE 1

Effect of in vivo exposure of glabridin on the plasma levels of NOx and TNF-α and the nitrite production by peritoneal macrophages

Different groups ($n = 4$) of animals were treated with vehicle, LPS (200 μg/kg i.p.), or LPS (200 μg/kg i.p.) + glabridin (1, 3, or 10 mg/kg at 24 and 2 h before LPS treatment). Whole blood samples were withdrawn from mice, and plasmas were prepared. The concentration of NOx and TNF-α were determined as described under *Materials and Methods*. Peritoneal macrophages were isolated 6 h after LPS treatment and cultured for 24 h. Culture supernatants were subsequently isolated and analyzed for nitrite production. Each column shows the mean ± S.D. of quadruplicate determinations. Significance was determined using Student's t test versus the control group.

	Plasma NOx	Plasma TNF-α	Nitrite
	μM	ng/ml	$nmol/10^6$ cells
Vehicle	0.38 ± 0.13	0.11 ± 0.02	1.13 ± 0.52
LPS (200 μg/kg)	6.03 ± 0.66	1.60 ± 0.39	31.87 ± 1.02
LPS (200 μg/kg) + glabridin (1 mg/kg)	5.69 ± 1.09	1.23 ± 0.44	30.25 ± 3.03
LPS (200 μg/kg) + glabridin (3 mg/kg)	3.60 ± 0.49*	1.08 ± 0.06	19.33 ± 2.35*
LPS (200 μg/kg) + glabridin (10 mg/kg)	1.51 ± 0.67*	0.73 ± 0.09*	4.37 ± 0.81*

* $p < 0.05$.

pathogenesis of septic shock and subsequent multiple organ dysfunction, and the induction of iNOS gene has been proposed to be a major factor involved in the pathologic vasodilation and tissue damage observed (Thiemermann, 1997; Titheradge, 1999). In addition, Gomez-Jimenez and coworkers demonstrated a quantitative and qualitative clinical relationship between NO production, endotoxemia, hemodynamic dysfunction, and multiple organ dysfunction in human septic shock (Gomez-Jimenez et al., 1995). In this report, we showed the protective effect of glabridin on LPS-induced sepsis, suggesting in vivo anti-inflammatory activity of glabridin. Further studies also demonstrated that the increase in plasma levels of NOx and TNF-α by LPS was down-regulated by glabridin treatment. Considering a critical role of inflammatory mediators, such as NO and TNF-α, in septic shock, our results suggest that increased survival of mice by glabridin treatment in animal model of sepsis might be mediated by inhibiting the production of NO and TNF-α.

NF-κB/Rel is a pleiotropic regulator of various genes involved in immune and inflammatory responses. Moreover, the inducibility of iNOS by LPS has been already shown to be primarily dependent on the transcription factor NF-κB/Rel (Xie et al., 1994). Pretreatment of RAW 264.7 cells with glabridin interfered with NF-κB/Rel DNA binding activity and NF-κB/Rel-dependent reporter gene expression, which was thought to be associated with the inhibitory effect of this agent on iNOS gene expression. As mentioned earlier, ubiquitous degradation of IκBs and subsequent nuclear translocation of p65 subunit of NF-κB/Rel are key regulatory steps in NF-κB/Rel activation (Shishodia and Aggarwal, 2004). Here, we clearly demonstrated that LPS-induced degradation of IκBα and IκBβ was inhibited by glabridin in RAW 264.7 cells without affecting the IKKα expression level. Consistent with this result, nuclear translocation of NF-κB/Rel p65 subunit was also down-regulated by glabridin. From these results, it is assumed that prevention of IKK activity can be a mechanism underlying the inhibitory effect of glabridin on NF-κB/Rel activation.

Various intracellular signaling pathways are involved in the modulation of NF-κB/Rel activity and inflammatory cytokine expression. Among various signaling pathways, mitogen-activated protein kinase pathway has been known to be important in the regulation of cytokine gene expression and NF-κB/Rel activation. It was reported that p38 MAP kinase and SAPK/JNK are important for iNOS gene expression (Chan and Riches, 1998; Jeon et al., 2000). Moreover, Nakano and coworkers reported that mitogen-activated protein kinase pathway is important for the activation of NF-κB/Rel (Nakano et al., 1998). We also previously demonstrated that SB203580, a specific inhibitor of p38 MAP kinase, inhibits LPS-induced NF-κB/Rel activation in RAW 264.7 cells (Jeon et al., 2000). Therefore, we investigated the effect of glabridin on LPS-induced phosphorylation of p38 MAP kinase and SAPK/JNK in RAW 264.7 cells; however, no significant changes by glabridin in the LPS-induced activation of MAPKs were observed. This result suggests that mitogen-activated protein kinases are not involved in the inhibitory effect of glabridin on LPS-induced iNOS gene expression and NF-κB/Rel activation.

We searched another possible target that can lead to the inhibition of IKK activity and activation of NF-κB/Rel and hypothesized that ROS pathway might be responsible for the inhibitory effect of glabridin on NF-κB/Rel activation. It is well known that ROS pathway regulate the NF-κB/Rel activation (Flohe et al., 1997). Moreover, a recent report of Sanlioglu and coworkers demonstrated that changes in intracellular ROS can regulate signal transduction pathway leading to modulation of IKK and NF-κB/Rel activity (Sanlioglu et al., 2001). In addition, glabridin was previously known to have antioxidant properties and inhibits low-density lipoprotein oxidation (Vaya et al., 1997; Belinky et al., 1998). In the present study, we demonstrated that glabridin has an intracellular radical scavenging activity in RAW 264.7 cells, suggesting a possible mechanism responsible for the inhibitory effect of glabridin on NF-κB/Rel activation. Thus, it is assumed that glabridin inhibits NF-κB/Rel activation, at least in part, by inhibiting ROS generation. As shown in our results, nuclear translocation of p65 and following events were not significantly affected by 1 μM glabridin; however, in contrast to other results, IκB degradation and ROS production was inhibited by 1 μM glabridin. Considering that ROS regulates IKK activity and IκB degradation, it is assumed that the discrepancy in dose response occurs between IκB degradation and NF-κB/Rel activation. Other mechanisms which block the inhibitory effect of 1 μM glabridin may be involved in this process, and increased amounts of glabridin may be needed to overcome this. Further studies are required to fully elucidate the mechanism of NF-κB/Rel inhibition by glabridin.

In summary, we demonstrated that glabridin inhibits LPS-induced NO production and iNOS gene expression in macrophages and that these effects are mediated, at least in part, by blocking NF-κB/Rel activation. Our results also showed that the inhibitory effect of glabridin on NF-κB/Rel activation was due to the inhibition of ROS pathway. Moreover, glabridin exerted potent in vivo anti-inflammatory effects and in-

creased the survival rate of mice in sepsis model. The results presented in this report give an insight into the mechanism responsible for various pharmacological activity of glabridin and licorice extract.

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Address correspondence to: Hwan Mook Kim, Bioevaluation Center, Korea Research Institute of Bioscience and Biotechnology, Taejon, 305-333, Korea. E-mail: hwanmook@kribb.re.kr