Nodakenin Suppresses Lipopolysaccharide-Induced Inflammatory Responses in Macrophage Cells by Inhibiting Tumor Necrosis Factor Receptor-Associated Factor 6 and Nuclear Factor-κB Pathways and Protects Mice from Lethal Endotoxin Shock

Hong-Kun Rim, Woong Cho, Sang Hyun Sung, and Kyung-Tae Lee

Departments of Pharmaceutical Biochemistry (H.-K.R., W.C., K.-T.L.) and Life and Nanopharmaceutical Science (W.C., K.-T.L.), College of Pharmacy, and Department of Biomedical Science, College of Medical Science (H.-K.R., K.-T.L.), Kyung Hee University, Seoul, Republic of Korea; and College of Pharmacy, Seoul National University, Seoul, Republic of Korea (S.H.S.)

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ABSTRACT

Nodakenin, a coumarin isolated from the roots of Angelicae gigas, has been reported to possess neuroprotective, anti-gregatory, antibacterial, and memory-enhancing effects. In the present study, we investigated the anti-inflammatory effects of nodakenin by examining its in vitro inhibitory effects on inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and proinflammatory cytokines in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages and mouse peritoneal macrophages and its in vivo effects on LPS-induced septic shock in mice. Our results indicate that nodakenin concentration-dependently inhibits iNOS and COX-2 at the protein, mRNA, and promoter binding levels, and these inhibitions cause attendant decreases in the production of nitric oxide (NO) and prostaglandin E2 (PGE2). Furthermore, we found that nodakenin inhibits the production and mRNA expression of tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β induced by LPS. Molecular data revealed that nodakenin suppressed the transcriptional activity and translocation of nuclear factor-κB (NF-κB) by inhibiting inhibitory κB-α degradation and IκB kinase-α/β phosphorylation. In addition, nodakenin was found to significantly inhibit the LPS-induced binding of transforming growth factor-β-activated kinase 1 to tumor necrosis factor-receptor-associated factor 6 (TRAF6) by reducing TRAF6 ubiquitination. Pretreatment with nodakenin reduced the serum levels of NO, PGE2, and proinflammatory cytokines and increased the survival rate of mice with LPS-induced endotoxemia. Taken together, our data suggest that nodakenin down-regulates the expression of the proinflammatory iNOS, COX-2, TNF-α, IL-6, and IL-1β genes in macrophages by interfering with the activation of TRAF6, thus preventing NF-κB activation.

INTRODUCTION

The stimulation of Toll-like receptor 4 by lipopolysaccharide (LPS) promotes the recruitment of myeloid differentiation factor 88/interleukin (IL)-1 receptor-associated kinase (IRAK)/tumor necrosis factor receptor-associated factor 6 (TRAF6) and results in the activation of transforming growth factor-β-activated kinase 1 (TAK1) in macrophages (Wang et al., 2001). Activated TAK1 subsequently induces the activation of transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), by phosphorylating the inhibitor of κB (IκB) kinase complex (IKK) and/or mitogen-activated protein kinase (MAPK) (Lee et al., 2000; Sakurai et al., 2002). These activated transcription factors then regulate

ABBREVIATIONS: LPS, lipopolysaccharide; AP-1, activator protein-1; Con, control; COX, cyclooxygenase; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-related kinase; IκB, inhibitor of κB; IKK, IκB kinase; IL, interleukin; IRAK1, IL-1 receptor-associated kinase 1; JNK, c-Jun N-terminal kinase; L-NIL, L-NAME-(1-iminoethyl) lysine; MAPK, mitogen-activated protein kinase; MITT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, NO synthase; iNOS, inducible NOS; p-, phosphorylated; NS398, N-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PG, prostaglandin; RT, reverse transcriptase; TRAF1, transforming growth factor-β-activated kinase 1; TNF-α, tumor necrosis factor-α; TRAF6, tumor necrosis factor receptor-associated factor 6; Ub, ubiquitin; Ubc, Ub-conjugating enzyme complex.
various proinflammatory mediator and cytokine genes, including inducible nitric-oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), IL-6, and IL-1β (Wan and Lenardo, 2010).

Nitric oxide is a well-known proinflammatory mediator in the pathogenesis of inflammation (Vane et al., 1989). In mammalian cells, NO is synthesized by the three isoforms of NOS: neuronal NOS, endothelial NOS, and iNOS (Zhou and Zhu, 2008). Moreover, although neuronal and endothelial NOS are constitutively expressed, iNOS is expressed in response to interferon-γ, LPS, and various proinflammatory cytokines (Kim et al., 2007b). The NO produced by iNOS has been described to have beneficial antioxidant, microbicidal, and antitumoral effects (Kleinert et al., 2004), but the overproduction of NO can be harmful to the host and, in fact, is involved in the pathogenesis of various inflammatory diseases (Sharma et al., 2007). The promoter region of the gene encoding iNOS contains NF-κB binding motifs, and it has been shown that the binding of NF-κB to NF-κB sites upstream of iNOS promoter plays an important role in the LPS-induced up-regulation of the iNOS gene. Prostanoids are also important proinflammatory mediators and may act in a paracrine or autocrine manner to coordinate intracellular events (Smith et al., 2000). COX-1 and COX-2 convert arachidonic acid to prostaglandin (PG) H₂, the precursor of the prostanoid thromboxane A₂ and the PGs (PGD₂, PGE₂, PGF₂α, and PGL₂) (Kang et al., 2007). COX-1 is constitutively expressed in almost all tissues and supplies PGs to maintain physiological functions, whereas COX-2 expression is up-regulated in an inflammatory background and accounts for the excessive production of inflammatory PGs. In particular, PGE₂ is a major COX-2 product at inflammatory sites where it contributes to local blood flow increases, edema formation, and pain sensitization (Prescott and Fitzpatrick, 2000; Hinz and Brune, 2002).

It has been reported that the roots of Angelicae gigas Nakai (Umbelliferae) exhibit various pharmacological effects, such as cancer preventative and antioxidative effects (Heo et al., 2007; Lv et al., 2007; Yoo et al., 2007). Decursin, a major coumarin component in A. gigas, has been reported to inhibit the expression of matrix metalloproteinase-9 and cytokines via the NF-κB pathway, and derivatives of decursin have been reported to have inhibitory effects on lung inflammation (Kim et al., 2006; Yang et al., 2009). In addition, five coumarin compounds (decursin, decursinol angelate, 7-dimethylsulfoxide, marmesin, and decursinol) have been reported to suppress levels of iNOS, IL-1β, and COX-2 in LPS-induced RAW 264.7 macrophages effectively (Ma et al., 2009). Nodakenin, a furanocoumarin glycoside isolated from the dried roots of A. gigas, has been reported to possess neuroprotective, antiaggregatory, antibacterial, and memory-improving effects (Lee et al., 2003a,b; Kang and Kim, 2007; Kim et al., 2007a). However, no report has been issued on its anti-inflammatory activities or the molecular mechanisms involved. Therefore, as a part of our ongoing screening program to evaluate the anti-inflammatory potentials of natural compounds, we investigated the molecular mechanisms underlying the anti-inflammatory properties of nodakenin in activated macrophages and a murine model of sepsis.

Materials and Methods

Reagents. Nodakenin (Fig. 1A) was kindly provided by Sang Hyun Sung (Seoul National University), and its purity (> 97%) was determined by high-performance liquid chromatography-mass spectrometry analyses (Ahn et al., 2008). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). COX-1, COX-2, extracellular signal-related kinase (ERK), IKK-α/β, IκB-α, iNOS, IRAK1, c-Jun N-terminal kinase (JNK), p65, p38, PARP, TRAF6, ubiquitin (Ub), and β-actin monoclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). p-IKK-α/β, p-IκB-α, p-p38, TAK1, and p-TAK1 monoclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA). Thioglycollate was obtained from Difco (Detroit, MI). Enzyme-linked immunosorbent assay (ELISA) kits for PGE₂, TNF-α, IL-6, and IL-1β were obtained from R&D Systems (Minneapolis, MN). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide, sulfanilamide, phenylmethylsulfonylfluoride, dithiothreitol, l-Nω-(1-iminoethyl)lysine (l-NIL), LPS, sodium bicarbonate, HEPES, SDS, and all other chemicals were purchased from Sigma (St. Louis, MO).

Cell Culture and Sample Treatment. RAW 264.7 macrophages were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea). Cells were cultured in DMEM containing 10% fetal bovine serum, penicillin, and streptomycin sulfate at 37°C in a 5% CO₂ atmosphere. Mouse peritoneal macrophage cells were obtained 4 days after intraperitoneal injection of 2 ml of thioglycollate to the 10-week-old C57BL/6 male mice and isolated as reported previously (Alleva et al., 2002). Thioglycollate (4%), prepared according to the manufacturer’s instructions, was used to recruit macrophages to the peritoneal cavity of mice. The cells were washed twice, resuspended in DMEM, and seeded in culture plates. Cells were incubated with various concentrations of nodakenin (25, 50, or 100 μM) or positive chemicals and then stimulated with LPS (1 μg/ml) for the indicated times.

Animals. C57BL/6 male mice (6 or 10 weeks) were obtained from Dae-Han Biolink Co. (Eumsung-Gun, Chungbuk, Republic of Korea) and maintained under constant conditions (temperature, 20–25°C; humidity, 40–60%; 12-h light/dark cycle). At 24 h before the experiment, only water was provided. All procedures were conducted in accordance with university guidelines and approved by the ethical committee for Animal Care and the Use of Laboratory Animals, College of Pharmacy, Kyung Hee University (KHP-2010-11-4).

MTT Assay. Cells were incubated with a MTT solution for 4 h at 37°C under 5% CO₂. One hundred microliters of dimethyl sulfoxide was added to extract the MTT formazan, and the absorbance of each well at 540 nm was read by an automatic microplate reader (Molecular Devices, Sunnyvale, CA).

Nitrite Determination. The nitrite accumulated in the culture medium was measured as an indicator of NO production based on the Griess reaction. In brief, 100 μl of conditioned medium was mixed with an equal volume of Griess reagent [equal volume of 1% (v/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthyl-ethylene-diamine-HCl], incubated at room temperature for 15 min, and then incubated for 10 min, and then the absorbance at 540 nm was measured in a microplate reader (Molecular Devices). The amount of nitrite in the samples was determined by using NaNO₂ as a standard.

Determination of PGE₂, TNF-α, IL-6, and IL-1β Production. PGE₂, TNF-α, IL-6, and IL-1β levels in cell culture media were quantified by using ELISA kits according to the manufacturer’s instructions.

Western Blot Analysis. The cells were collected by centrifugation and washed once with phosphate-buffered saline (PBS). Washed cell pellets were resuspended in the protein extraction solution PRO-PREP (Intron Biotechnology, Seoul, Republic of Korea) and then incubated for 15 min at 4°C. Cell debris was removed by microcentrifugation, and supernatants were quick-frozen. The protein concentration was determined by using the Bio-Rad pro-
tein assay reagent (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instruction. Forty micrograms of protein were electroblotted onto a polyvinylidene difluoride membrane after separation on 10% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated for 1 h with blocking solution (5% skim milk) at room temperature, and then incubated overnight with a 1:1000 dilution of primary antibody at 4°C. Blots were washed three times with Tween 20/Tris-buffered saline and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa
Cruz, CA) for 2 h at room temperature. Blots were again washed three times with Tween 20/Tris-buffered saline and then developed by enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

**Immunoprecipitation.** Cells were harvested and washed twice with cold PBS. The cell pellets were resuspended and lysed in EBC buffer (50 mM Tris, pH 8.0, 120 mM NaCl, 0.5% nonidet P-40, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 50 μg/ml phenylmethylsulfonyl fluoride, 0.2 mM sodium ortho-vanadate, and 100 mM NaF) for 30 min at 4°C. After centrifugation (10,000 g, 5 min), protein concentrations were determined. Equal amounts of protein (1000 μg) were incubated with antibodies for 12 h at 4°C, followed by incubation with 10 μl of protein A or protein G-magnetic beads for 4 h. The protein complex was washed four times with EBC buffer and released from the beads by boiling in sample buffer (350 mM Tris, pH 6.8, 10% SDS, 30% β-mercaptoethanol, 6% glycerol, and 0.12% bromophenol blue) for 5 min. The reaction mixture was then subjected to Western blot.

**RNA Preparation and RT-PCR.** Total cellular RNA was isolated by using Easy Blue Kits (Intron Biotechnology), according to the manufacturer's instructions. RNA (1 μg) was reverse-transcribed from each sample by using murine leukemia virus reverse transcriptase, 1 mM dNTP, and 0.5 μg/μl oligo(dT)12–18. PCR analyses were performed on aliquots of the cDNA preparations to detect iNOS, COX-2, TNF-α, IL-6, and IL-1β (using β-actin as an internal standard) gene expression by using a thermal cycler (PerkinElmer Life and Analytical Sciences, Waltham, MA). Reactions were carried out in a volume of 20 μl containing (final concentrations) 1 unit of Taq DNA polymerase, 0.2 mM dNTP, reaction buffer, and 10 pmol of 5′ and 3′ primers. PCR primers used in this study were purchased from Bioneer (Seoul, Republic of Korea): iNOS, 5′-ATG TGC AGT AGA CTT TTA CA-3′; 5′-ACT CAG CGA GAA CTC-3′, TNF-α, 5′-ATG AGC ACA GCA AGC AGT ATC-3′, 5′-TAC AGG GAT ACC ACT CCC AAC AGA CC-3′ (Bioneer, Seoul, Republic of Korea); and COX-2 promoter plasmid (pGL3-COX-2; Promega, Madison, WI) by using Lipofectamine LTX (Invitrogen, Carlsbad, CA) as instructed by the manufacturers. At 24 h after transfection, cells were pretreated with nodakenin for 1 h and stimulated with 1 μM LPS. After 24 h of stimulation, cells were lysed, and the luciferase activity was determined by using the Promega luciferase assay system (Promega) and a luminometer (PerkinElmer Life and Analytical Sciences).

**Septic Shock Model.** The septic shock model test was performed as reported previously (Chaudhry et al., 2008). The C57BL/6 mice were injected intraperitoneally with PBS or LPS (25 mg/kg) dissolved in PBS. Nodakenin (10 or 20 mg/kg) was injected intraperitoneally for 1 h before LPS injection. Survival was monitored for 72 h. Four hours after LPS injection, peripheral blood samples were obtained from each mouse. NO and PGE2 levels in serum were measured by using Griess reagents and ELISA kits, respectively. Serum levels of the inflammatory cytokines (TNF-α, IL-6, and IL-1β) were quantitated by using mouse Bio-Plex pro assays (Bio-Rad Laboratories), according to the manufacturer's instructions.

**Statistical Analysis.** All data are presented as means ± S.D. Statistically significant values were compared by using analysis of variance and Dunnett's post hoc test. Survival data were analyzed with the Kaplan-Meier method and log rank test. Statistical significance was set at P < 0.05.

**Results**

**Nodakenin Inhibited LPS-Induced NO and PGE2 Production by the Suppression of iNOS and COX-2 Expression in RAW 264.7 Macrophages.** To investigate whether nodakenin has anti-inflammatory activities, NO and PGE2 production was determined in the presence of nodakenin at 25, 50, or 100 μM in LPS-induced RAW 264.7 macrophages. LPS-induced nitrite (a surrogate of NO production) and PGE2 generation were significantly and concentration-dependently attenuated by nodakenin, from concentrations as low as 25 μM (Fig. 1, B and C). L-NIL (10 μM) and N-(2-cyclohexoxy-4-nitrophenyl)methanesulfonamide (NS398) (4 μM) were used as a positive control inhibitors. The inhibition of the production of NO and PGE2 by nodakenin can result from the suppression of the enzymic activities and/or expression levels of iNOS and COXs. Initially, we investigated whether the enzymic activities of iNOS, COX-1, and COX-2 were affected by nodakenin, but we found that nodakenin did not reduce the production of NO or PGE2 by enzyme-based iNOS and COX-catalytic activity determinations (data not shown). Next, we investigated whether inhibition of the production of NO and PGE2 by nodakenin is related to reductions in the expression of iNOS, COX-1, or COX-2. We found LPS-induced iNOS and COX-2 protein and mRNA levels were significantly inhibited in nodakenin in a concentration-dependent manner (Fig. 1, D and E), but nodakenin had no effect on the expression of COX-1 protein in RAW 264.7 macrophages (Fig. 1D). When we analyzed the transcriptional activities of iNOS and COX-2 gene promoters, LPS was found to enhance the activities of both significantly, and nodakenin was found to inhibit these inductions concentration-dependently. In addition, these inhibitory effects of nodakenin were not caused by nonspecific cytotoxicity, because nodakenin had no effect on cell viability as determined by MTT assay at concentrations from 25 to 100 μM (data not shown).

**Nodakenin Suppressed the LPS-Induced Expression of TNF-α, IL-6, and IL-1β in RAW 264.7 Macrophages.** Next, we examined TNF-α, IL-6, and IL-1β production and mRNA expression in LPS-induced macrophages pretreated with nodakenin. Pretreatment with nodakenin was found to reduce the LPS-induced production of TNF-α, IL-6, and IL-1β (Fig. 2, A–C) and their mRNA expression (Fig. 2D) concentration-dependently, showing that nodakenin suppresses the expression of these inflammatory genes at the transcriptional level. Collectively, these results indicate that nodakenin could inhibit the expression of iNOS and COX-2 and other proinflammatory genes.

**Nodakenin Inhibited the LPS-Induced Production of NO, PGE2, and Proinflammatory Cytokines in Mouse Peritoneal Macrophages.** To determine whether the anti-inflammatory effects of nodakenin observed in RAW 264.7 macrophages also occur in primary cells, we examined the effect of nodakenin on the LPS-induced production of NO, PGE2, TNF-α, IL-6, and IL-1β in peritoneal macrophages isolated from C57BL/6 mice. In these cells, nodakenin was found to inhibit the LPS-induced production of NO, PGE2,
and proinflammatory cytokines significantly and concentration-dependently (Fig. 3, A–C) and reduce the protein levels of iNOS and COX-2 and the mRNA levels of iNOS, COX-2, and proinflammatory cytokines (TNF-α, IL-6, and IL-1β) in a concentration-dependent manner (Fig. 3, D and E). We confirmed that these inhibitory effects of nodakenin were not caused by a toxic effect.

**Nodakenin Inhibited LPS-Induced NF-κB Activation in RAW 264.7 Macrophages.** Because the activation of NF-κB and AP-1 is critically required for the LPS-induced transcriptional regulation of inflammation (Sakurai et al., 2002), we examined the effect of nodakenin on LPS-induced NF-κB- and AP-1-dependent reporter gene activities. Analysis of reporter gene expression using pNF-κB-luc or pAP-1-luc demonstrated that nodakenin concentration-dependently inhibited NF-κB-dependent luciferase activity, but did not affect AP-1-dependent luciferase activity (Fig. 4A). In general, the translocation of transcription factors into the nucleus is believed to be essentially required for the transcriptional activations of target genes. Accordingly, we investigated whether nodakenin prevents the nuclear translocation of the p65 subunit of NF-κB. It was found that pretreatment with nodakenin concentration-dependently attenuated the LPS-induced nuclear translocation of p65 (Fig. 4B).

**Effects of Nodakenin on the LPS-Induced Phosphorylation of IkB-α, IKK, and MAPKs.** In its inactive state NF-κB binds to its inhibitor protein, IkB-α, in the cytoplasm, but after cellular stimulation IkB-α is phosphorylated at specific serine residues and undergoes polyubiquitination and proteasomal degradation, which free NF-κB, allowing it to be translocated to the nucleus (Hatada et al., 2000). Thus, we explored whether nodakenin inhibits the LPS-induced phosphorylation and degradation of IkB-α in RAW 264.7 macrophages. Figure 5A shows that the LPS-induced phosphorylation and degradation of IkB-α were significantly blocked by nodakenin pretreatment. In addition, because IKK-α and IKK-β are upstream kinases of IkB in the NF-κB signal pathway, we examined the effects of nodakenin on the LPS-induced activation of IKK-α/β in RAW 264.7 macrophages by Western blotting. We found nodakenin markedly reduced LPS-induced IKK-α/β phosphorylation but did not affect the total amounts of IKK-α or IKK-β (Fig. 5B).

Several MAPKs, including p38, JNK, and ERK1/2, are involved in the signal transduction pathways that lead to the regulation of inflammatory mediators. Moreover, these pathways play critical roles in the activation of NF-κB (Rajapakse et al., 2008). Therefore, we investigated the effects of nodakenin on the LPS-induced phosphorylation of MAPKs in RAW 264.7 macrophages by Western blotting. As shown in Fig. 5C, nodakenin concentration-dependently suppressed the LPS-induced activation of p38, but did not affect the phosphorylation of JNK or ERK1/2. However, the levels of nonphosphorylated p38, JNK, and ERK1/2 were unaffected by LPS or nodakenin. These results indicate that p38 phosphorylation may be involved in the inhibition of LPS-induced inflammatory responses in RAW 264.7 macrophages by nodakenin.

**Nodakenin Suppressed TAK1 and TRAF6 Signaling in LPS-Induced RAW 264.7 Macrophages.** Because TAK1 has been implicated in the regulation of IKK-α/β and/or MAPK phosphorylation by LPS (Wang et al., 2001), we examined whether nodakenin could inhibit the LPS-induced phosphorylation of TAK1 in RAW 264.7 macrophages. As shown in Fig. 6A, LPS-induced TAK1 phosphorylation was...
significantly blocked by nodakenin in a concentration-dependent manner. Because binding of TAK1 to TRAF6 is a crucial step in the activation of TAK1, we immunoprecipitated TAK1 by using specific antibodies and then detected bound TRAF6 by Western blotting. Treatment with nodakenin significantly inhibited binding between TAK1 and TRAF6 (Fig. 6B). Because ubiquitination of TRAF6 (by Lys-63 ligation) is known to be required for the activation of TAK1 (Walsh et al., 2008), we investigated whether nodakenin had an effect on LPS-induced ubiquitination in RAW 264.7 macrophages. Cell lysates were immunoprecipitated by using anti-TRAF6 antibody, and then the levels of conjugated ubiquitin in immune complexes were determined by Western blotting. LPS-induced ubiquitination of TRAF6 was significantly reduced by nodakenin at 10 min (Fig. 6C). However, TRAF6 protein levels were unaffected by LPS or nodakenin. Because nodakenin inhibited the LPS-induced ubiquitination of TRAF6, we further investigated the possibility that the upstream signaling protein IRAK1 might be involved in the activation of TRAF6. Immunoblot analysis showed that treatment with nodakenin did not prevent the LPS-induced degradation of IRAK1 in RAW 264.7 macrophages (Fig. 6D). These results suggest that nodakenin inhibits LPS-induced signal transduction by inhibiting the ubiquitination of TRAF6, thus preventing the association between TRAF6 and TAK1 and the subsequent activation of TAK1.

Nodakenin Inhibited the In Vivo Production of NO, PGE₂, and Proinflammatory Cytokines and Protected Mice from LPS-Induced Septic Death.

NO and proinflammatory cytokines play pivotal roles in the pathogenesis of sepsis (Opal, 2007). In view of the ability of nodakenin to attenuate LPS-induced NO, PGE₂, and proinflammatory cytokine levels, we investigated the effects of nodakenin in an animal model of sepsis. LPS administration (25 mg/kg i.p.) markedly increased serum levels of NO, PGE₂, TNF-α, IL-6, and IL-1β, but pretreatment with nodakenin (10 or 20 mg/kg i.p.) 1 h before LPS administration significantly decreased the production of NO, PGE₂, and these three proinflammatory cytokines (Fig. 7, A–E). Subsequently, we observed that nodakenin improved survival during sepsis. As shown in Fig. 7F, LPS (25 mg/kg i.p.) resulted in 100% mortality at 40 h postinjection, but pretreatment with nodakenin (10 or 20 mg/kg i.p.) significantly increased the survival rate.
Fig. 4. Effects of nodakenin on LPS-induced NF-κB activity and its translocation into the nucleus in RAW 264.7 macrophages. A, cells were transiently transfected with pNF-κB-luc reporter or pAP-1-luc reporter; phRL-TK vector was used as an internal control. Cells were pretreated with/without the indicated concentrations of nodakenin for 1 h and then stimulated with LPS (1 μg/ml) for 24 h. Cells were then harvested, and luciferase activity levels were determined as described under Materials and Methods. Controls were not treated with LPS or nodakenin. B, cells were pretreated with/without the indicated concentrations of nodakenin for 1 h and then stimulated with LPS (1 μg/ml) for 1 h. Nuclear (n) and cytosolic (c) extracts were isolated, and levels of p65 in each fraction were determined by Western blot. PARP and β-actin were used as internal controls. Density ratios versus PARP or β-actin were measured by densitometry. The experiment was repeated three times, and similar results were obtained. Data are presented as means ± S.D. of three independent experiments. #, P < 0.05 versus the control group. *, P < 0.05; **, P < 0.01 versus the LPS-treated group.

mg/kg i.p.) reduced this lethality to 80 or 50%, respectively, at 72 h postinjection.

Discussion

In the present study, we describe for the first time the anti-inflammatory activity of nodakenin in vitro and in vivo. Furthermore, we report that nodakenin inhibits the activation of TRAF6/TAK1/NF-κB and the subsequent induction of proinflammatory mediators, such as NO, PGE₂, TNF-α, IL-6, and IL-1β, and protects mice from lethal endotoxin shock.

The pathology of inflammation is initiated by complex processes triggered by microbial pathogens or their antigens (Caivano et al., 2003). The prototypic endotoxin LPS can potently activate macrophages and induce various proinflammatory mediators (Martinez et al., 2008); therefore, reducing activation signals in activated macrophages has been suggested as a therapeutic strategy in various inflammatory diseases (Duffield, 2003). In the present study, we found that nodakenin inhibited the LPS-induced production of NO and PGE₂ by suppressing the transcription of iNOS and COX-2 and thus their protein expression. The anti-inflammatory effects of nodakenin are supported by the results of our investigations of its effects on several proinflammatory cytokines, namely, TNF-α, IL-6, and IL-1β in LPS-induced macrophages, because they are known to have profound effects on the regulation of immune reactions, hematopoiesis, inflammation, and, in some cases, shock and death (Christiaens et al., 2008). More specifically, nodakenin was found to inhibit LPS-induced increases in the production and mRNA expression of TNF-α, IL-6, and IL-1β.

Of the multitude of transcription factors involved in intracellular signaling pathways, NF-κB is known to regulate genes involved in cell survival and coordinate the expression of proinflammatory mediators (Prescott and Fitzpatrick, 2000; Doyle and O’Neill, 2006). In accord with these findings, the inhibition of NF-κB has been shown to be effective at controlling inflammatory diseases in several animal models (Li and Verma, 2002; Shibata et al., 2007). Accordingly, in the present study, we examined whether nodakenin inhibits NF-κB activity in RAW 264.7 macrophages by using reporter gene assays and found that nodakenin inhibited the LPS-induced transcriptional activity of NF-κB in a concentration-dependent manner. Based on these results, we suggest that the inhibition of the expression of proinflammatory mediators by nodakenin might be caused by the suppression of NF-κB activation. To identify the mechanisms involved in the inhibition of NF-κB activity by nodakenin, we tested the effect of nodakenin on NF-κB activation signals. In the present study, nodakenin was found to inhibit the LPS-induced phosphorylation and degradation of IkB-α and reduce the amount of p65 in nuclear fractions. These findings suggest that the inhibition of NF-κB activation by nodakenin is the result of the inhibition of IkB-α phosphorylation and degradation and thus of the nuclear translocation of p65.

Multiple lines of evidence have demonstrated that the phosphorylation of IkB is regulated by IKK-α/β (Mercurio et al., 1997; Régnier et al., 1997), which is further regulated by upstream factors, such as TAK1 (Wang et al., 2001). These kinases may represent novel sites for pharmacological intervention in a number of inflammatory conditions. In the present study, it was found that nodakenin inhibited the phosphorylation of IKK-α/β and TAK1, suggesting that nodakenin suppresses NF-κB activation via the down-regulation of the TAK1-mediated NF-κB pathway in LPS-induced RAW 264.7 macrophages. TAK1 can phosphorylate MAPKs as well as IKK complex (Ninomiya-Tsuji et al., 1999), and the MAPK cascade is activated by LPS binding and contributes to the production of proinflammatory cytokines (Buckley et al., 2006). Furthermore, MAPK phosphorylation activates the transcription of NF-κB-mediated proinflammatory cytokines (Rajapakse et al., 2008); thus, MAPKs are viewed as targets for novel anti-inflammatory drugs. In vitro studies using RAW 264.7 macrophages
have shown that p38 is involved in NF-κB activation (Cho et al., 2008), whereas JNK controls AP-1 activation (Shan et al., 2009). In the present study, nodakenin was found to exclusively regulate p38 phosphorylation in LPS-induced RAW 264.7 macrophages, which supports the idea that nodakenin affects proinflammatory signaling not through AP-1 activation via JNK but through NF-κB activation via p38, as has been reported for chemopreventive phytochemicals, such as diarylheptanoid (Yadav et al., 2003), capsaicin (Chen et al., 2003), and sesquiterpene lactone (Cho et al., 2009), which were found to inhibit iNOS and COX-2 by targeting MAPK signaling pathways, including the p38 kinase pathway.

TRAF6, a member of the TRAF family, is a Lys-63-specific E3 ubiquitin ligase and has a RING finger domain. Unlike other TRAFs, TRAF6 has a unique C-terminal domain that facilitates oligomerization and binding with IRAK1 (Walsh et al., 2008). When LPS binds to Toll-like receptor 4, IRAK1 oligomerizes and interacts with TRAF6, which then oligomerizes and activates ligase activity to promote autoubiquitination by the Ub-conjugating enzyme complex (Ubc13-Uev1A). TRAF6 in conjunction with Ubc13-Uev1A catalyzes the formation of a unique poly-Ub chain linked through Lys-63 of Ub (Dong et al., 2006). Furthermore, specific modification of TRAF6 by ubiquitination recruits the TAK1/TAK1 binding protein complex and results in TAK1 activation (Walsh et al., 2008). Therefore, we investigated the effect of nodakenin on TRAF6-TAK1 binding and TRAF6 ubiquitination. Western blot analysis and immunoprecipitation studies revealed that nodakenin inhibited TRAF6-TAK1 binding and TRAF6 ubiquitination, which is one of the key components of LPS-mediated TAK1/IKK/NF-κB upstream signaling. It has been reported that eupatolide inhibits the expression of iNOS and COX-2 by inducing the proteasomal degradation of TRAF6 in LPS-stimulated RAW 264.7 macrophages (Lee et al., 2010). However, in the present study, nodakenin did not affect TRAF6 at the protein level, but rather decreased the ubiquitination of TRAF6 in RAW 264.7 macrophages.

Upon LPS stimulation, IRAK1 is phosphorylated within receptor complexes, such as myeloid differentiation factor 88, IRAK4, and TRAF6. Phosphorylated IRAK1 then triggers TRAF6 ubiquitination and its translocation to the TAK1/TAK1 binding protein complex, and phosphorylated IRAK1 is then ubiquitinated and degraded at proteasomes (Conze et al., 2008). Therefore, we examined the effect of nodakenin on the LPS-induced degradation of IRAK1. However, we found that the LPS-induced degradation of IRAK1 was not blocked by nodakenin. These results suggest that nodakenin inhibits LPS-induced inflammatory mediator expression by inhibiting TRAF6 ubiquitination in RAW 264.7 macrophages, thus suppressing the LPS-stimulated activation of TAK1/IKK-α/β/NF-κB and p38 MAPK. Although the present study does not clarify the mechanism responsible for the inhibition of TRAF6 ubiquitination, Western blot analysis and immunoprecipitation studies revealed that nodakenin inhibited TRAF6-TAK1 binding and TRAF6 ubiquitination, which is one of the key components of LPS-mediated TAK1/IKK/NF-κB upstream signaling. It has been reported that eupatolide inhibits the expression of iNOS and COX-2 by inducing the proteasomal degradation of TRAF6 in LPS-stimulated RAW 264.7 macrophages (Lee et al., 2010). However, in the present study, nodakenin did not affect TRAF6 at the protein level, but rather decreased the ubiquitination of TRAF6 in RAW 264.7 macrophages.

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Fig. 5. Effects of nodakenin on the LPS-induced phosphorylation of IκB-α (A), IKKs (B), and MAPKs (C) in RAW 264.7 macrophages. Cells were pretreated with/without nodakenin (25, 50, or 100 μM) for 1 h and then stimulated with LPS (1 μg/ml) for 15 min. Total proteins were prepared, and Western blot was performed by using specific antibodies. Density ratios versus β-actin or total form were measured by densitometry. The experiment was repeated three times, and similar results were obtained. Data are presented as means ± S.D. of three independent experiments. #, P < 0.05 versus the control group. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus the LPS-treated group.
uitation by nodakenin, we suggest that nodakenin could act at the C-terminal domain of TRAF6 at the sites that interact with IRAK1 or Ubc13-Uev1A.

To verify the in vivo relevance of our in vitro results regarding the anti-inflammatory effects of nodakenin, we evaluated its effects in a murine model of LPS-induced sepsis. LPS is the most frequent cause of sepsis, and circulating NO, proinflammatory cytokines, and chemokines frequently are elevated in sepsis. Furthermore, the modulation of these elevated NO and proinflammatory cytokine levels is an important therapeutic strategy in sepsis (Dinarello, 1997; Van Amersfoort et al., 2003). Pretreatment with nodakenin was found to reduce the serum levels of NO, PGE2, TNF-α, IL-6, and IL-1β and increase the survival rates of animals with established LPS-induced endotoxemia. These results suggest that the suppressive effects of nodakenin on NF-κB-regulated gene transcription in macrophages had an anti-inflammatory effect in our animal model of sepsis. On the other hand, it has been reported that coagulation is an important hallmark of sepsis and is associated with increased mortality (Amaral et al., 2004). Therefore, it is believed that the antiaggregatory (Lee et al., 2003b) and COX-2 inhibitory effects of nodakenin promote survival in septic mice. However, Schildknecht et al. (2004) cautioned against the use of COX-2-specific inhibitors in sepsis, because the inhibition of COX-2 enzymatic activity might increase prothrombotic activity. This study shows that the anti-inflammatory effects of nodakenin are caused by the inhibition of COX-2 expression and the transcriptional regulations of inflammatory genes. Collectively, these results indicate that the inhibitory effects of nodakenin on COX-2 expression do not make a significant contribution to the lethality in mice injected with LPS.

Although the anti-inflammatory effects of furanocoumarin have been shown to be caused by the inhibition of the production of PGE2 and NO (Wang et al., 2000; Ban et al., 2003), and furanocoumarin has greater inhibitory effects than simple coumarins and chromones (Wang et al., 1999), it is difficult to predict the anti-inflammatory activity of nodakinin, a linear furanocoumarin glycoside, from a structurally based perspective. Further pharmacological studies are needed to provide more detailed information on the structure-activity relationships exhibited by nodakenin derivatives.

In summary, our findings show that nodakenin inhibits the LPS-induced expression of inflammatory mediators by interfering with the ubiquitination of TRAF6 and the interaction between TRAF6 and TAK1, which in turn, blocks the activation of IKK and MAPK signal transductions and down-regulates NF-κB activation in RAW 264.7 macrophages. More importantly, the study shows that nodakenin...
decreases the serum levels of NO, PGE2, and inflammatory cytokines in vivo and protects mice from LPS-induced lethality. Thus, our findings indicate that nodakenin could be a useful pharmacologic tool for improving our understanding of basic cellular functions and suggest that nodakenin be considered for evaluation as a potential treatment option for inflammatory diseases.

Authorship Contributions

Participated in research design: Rim, Cho, Sung, and Lee.
Conducted experiments: Rim and Cho.
Contributed new reagents or analytic tools: Rim and Sung.
Performed data analysis: Rim, Cho, and Lee. Wrote or contributed to the writing of the manuscript: Rim, Cho, Sung, and Lee.

References


Fig. 7. Effects of nodakenin on LPS-induced septic shock in mice. A to E, male C57BL/6 mice (6 weeks) were injected with nodakenin (10 or 20 mg/kg; i.p.) (n = 7) or PBS as control (n = 7) for 1 h before LPS injection (25 mg/kg i.p.). Four hours after LPS injection, peripheral blood was obtained from each mouse. Serum levels of NO (A), PGE2 (B), TNF-α (C), IL-6 (D), and IL-1β (E) were then determined as described under Materials and Methods. Data are presented as means ± S.D. of each mouse. #, P < 0.05 versus the control group. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus the LPS-treated group. F, male C57BL/6 mice (6 weeks) were injected with nodakenin (10 or 20 mg/kg i.p.) (n = 10) or PBS as a vehicle (n = 10) 1 h before LPS injection (25 mg/kg i.p.). Survival was monitored for 72 h, and survival data were analyzed by using the Kaplan-Meier method and the log rank test.


